Université de Montréal

# Identification and isolation of plant promoters induced by thiocyanate

par

Zeina Nasr

Département de sciences biologiques

Faculté des arts et des sciences

Mémoire présenté à la Faculté des études supérieures en vue de l'obtention du grade de Maîtrise en sciences (M.Sc.) en sciences biologiques

Janvier, 2007

Université de Montréal
Faculté des études supérieures

Ce mémoire de maîtrise intitulé :

Identification and isolation of plant promoters induced by thiocyanate

présenté par :

Zeina Nasr

a été évalué par un jury composé des personnes suivantes:

| | |
|---|---|
| Président-rapporteur | Mohamed Hijri |
| Directeur de recherche | Hargurdeep S. Saini |
| Codirecteur | Daniel P. Maton |
| Membre du jury | Rajinder Dhindsa |

# ABSTRACT

Thiol methyl transferase (TMT) is an enzyme capable of methylating thiol compounds, such as thiocyanate ($^-$SCN), into their corresponding volatile methylated products. *TMT1* gene codes for TMT. Recently, *TMT1* was developed as a selectable marker. It was shown that *TMT1* is more efficient as a marker compared to the most used marker gene *nptII*. With the aim of developing *TMT1* as an inducible selectable marker that can be induced by one of the TMT substrates, $^-$SCN, a promoter was identified in *Arabidopsis thaliana* that might be able to induce *TMT1* expression only in the presence of $^-$SCN. The study was done on rice and *A. thaliana*. Plants were grown in the presence of $^-$SCN. In order to find the promoter, genes induced by $^-$SCN were identified by differential display analysis. Northern blot analysis confirmed that two genes were induced by the ion; the gene Os09g0442300 on chromosome 9 of rice that encodes a cysteine proteinase called oryzain γ, and the gene At1g03350 on chromosome 1 of *A. thaliana* that encodes a BSD domain-containing protein. For the rice gene, trials to isolate the upstream sequence that might contain the promoter region failed. In *A. thaliana*, a 502 bp sequence upstream of At1g03350 start codon was isolated and amplified. This region is expected to contain the $^-$SCN-inducible promoter. This region was introduced upstream of the reporter gene gusA. Further work is needed to measure the promoter activity in the presence and absence of $^-$SCN, and the promoter's ability to induce *TMT1* expression only in the presence of $^-$SCN.



**Keywords:** Thiol methyltransferase, thiocyanate, selectable marker, inducible promoter, differential display.

# RÉSUMÉ

La thiol méthyle transférase (TMT) est une enzyme capable de méthyler et détoxifier des ions toxiques, comme le thiocyanate ($^{-}$SCN). *TMT1* est un gène qui code pour la TMT. Récemment, *TMT1* a été utilisé comme un marqueur de sélection. *TMT1* a été montré d'être un marqueur plus efficace comparé à *nptII*, un marqueur le plus utilisé. Dans le but d'améliorer *TMT1* comme un marqueur de sélection inductible par $^{-}$SCN, c'est-à-dire, un marqueur qui ne devrait être exprimé que pendant le processus de sélection et en présence de $^{-}$SCN, un promoteur a été identifié chez l'*Arabidopsis thaliana*, qui pourrait induire l'expression de *TMT1* seulement en présence de $^{-}$SCN. L'étude a été faite sur le riz et l'*A. thaliana*. Afin de trouver le promoteur, des gènes induits par $^{-}$SCN on été identifiés par l'analyse différentielle des ARNs. L'analyse par Northern a confirmée la présence de deux gènes induits par l'ion; le gène Os09g0442300 sur le chromosome 9 du riz qui code pour une cystéine protéinase appelée oryzain γ, et le gène At1g03350 sur le chromosome 1 d'*A. thaliana* qui code pour une protéine contenant un domaine BSD. Pour le riz, on n'a pas réussi à isoler la séquence qui précède le gène et qui contient le promoteur inductible. Dans le cas du gène d'*A. thaliana,* une séquence de 502 paire de base qui précède le gène a été isolée et amplifiée. Cette région pourrait contenir le promoteur inductible. Elle a été insérée en amont du *gusA* pour mesurer l'activité du promoteur en présence et en absence de l'ion $^{-}$SCN. Ce travail permet d'étudier dans le future la capacité de ce promoteur d'induire l'expression de la *TMT1* seulement en présence de $^{-}$SCN.

**Mots clés:** Thiol méthyl transférase, thiocyanate, marqueur de sélection, promoteurs inductibles, analyse différentielle.

# TABLE OF CONTENTS

# LIST OF FIGURES

**Figure 26** Sequence of *A. thaliana* chromosome 1 showing the primers APF and APR.

# LIST OF TABLES

# ABBREVIATIONS

| | | |
|---|---|---|
| $^{-}$SCN | : | Thiocyanate ion |
| A260 | : | Absorbance reading at 260 nm |
| A280 | : | Absorbance reading at 280 nm |
| AdoMet | : | *S*-adenosyl-L-methionine |
| bp | : | base pair |
| cDNA | : | Complementary DNA |
| dATP | : | deoxyadenosine triphosphate |
| dCTP | : | deoxycytidine triphosphate |
| dGTP | : | deoxyguanosine triphosphate |
| dNTP | : | deoxyribonucleotide triphosphate |
| dTTP | : | deoxythymidine triphosphate |
| EDTA | : | Ethylenediaminetetra-acetic acid |
| GUS | : | beta-glucuronidase |
| H/BMT | : | halide/bisulfide methyltransferase |
| HPLC | : | High performance liquid chromatography |
| kb | : | kilobase |
| kDa | : | Kilo Dalton |
| LB | : | Luria-Bertani medium |
| MES | : | 2-(N-morpholino)ethanesulfonic acid |
| MOPS | : | 3-(N-morpholino)propanesulfonic acid |
| MS | : | Murashige and Skoog medium |
| MUG | : | 4-methylumbelliferyl β-D-glucuronide |
| NaClO | : | Sodium chlorite |
| *nptII* | : | neomycin phosphotransferase II |

| | | |
|---|---|---|
| PEG | : | Polyethylene glycol |
| SDS-PAGE | : | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| TBE | : | Tris-borate-EDTA |
| TEMED | : | N,N,N',N'-tetramethyl-ethylenediamine |
| TMT | : | Thiol methyltransferase |
| Tris | : | 2-amino-2-(hydroxylmethyl)-1,3-propanediol |
| Tween 20 | : | Polyoxyethylenesorbitan monolaurate |
| UV | : | Ultraviolet light |
| X-Gluc | : | 5-bromo-4-chloro-3-indolyl glucuronide |

# ACKNOWLEDGMENTS

I would like to express my gratitude to all those who gave me the possibility to complete this work. I am deeply indebted to my supervisor, Professor Deep H.S. Saini for his guidance, and moral and financial support during the course of this project. Words cannot describe my feelings. I can just say that it was an honor to have you as a supervisor and to feel to be a part of your family.

I would also like to express my sincere appreciation and respect for my co-supervisor, Professor Daniel Maton for his help and precious support during this work. I am also greatly thankful for my colleagues and dear friends in the lab, Dr. Priyum Koonjul for her constant and precious advice throughout these two years, Dr. Sima Babayeva for her help in the technical work, and Catherine Viel for her support in the beginning of this work. I would also like to thank Professor Daniel Maton's students for the support I got in their lab; special thanks to Martin, Hugo, and Eric for their help in the molecular work.

I would also like to thank every person working in the institute. It was a pleasure working with you. A special thanks to Fengshan Ma for his help and support at the end of this work.

Finally, I would like to thank my dearest Mom, Dad, Dana, and Nehme for their continual interest, support, and inspiration during these two years. For my parents, thank you for all what you've been through while I am away. For Dana, thank you for being my sister and my friend. For Nehme, thank you for listening and helping me in every possible way.

# DEDICATION

I dedicate this work to the most precious people in my life who offered me unconditional love and support during the course of this work: my great and amazing Mom, my best and dearest Dad, my wonderful sister and best friend Dana, and my love and my dear fiancé Nehme.

# 1. Introduction

## 1.1 Selectable markers: Definition and applications

Selectable markers are genes used to identify cells that are successfully transformed with a vector containing the gene of interest. The most common selectable markers are antibiotic-resistance genes (Miki and McHugh, 2004). Currently, neomycin phosphotransferase II (*nptII*) is the most widely used marker (Miki and McHugh, 2004). It confers resistance to neomycin and kanamycin. Following transformation with a vector containing both *nptII* and the gene of interest, only the cells that have integrated the genetic material are able to grow in a neomycin or kanamycin-containing medium. Other types of marker genes include herbicide tolerant genes, metabolic/auxotrophic genes and screenable markers. The first confers resistance to herbicides in a manner similar to antibiotic-resistance genes. The second allows the transformed plants to synthesize an important component for its survival that the wild type plants can not synthesize, so only transformed plants can survive and grow in a medium that lacks this component. The last type of marker genes code for proteins that can be identified through experimental procedures, so only transformed cells contain a specific protein. This type of markers are the least commonly used because selection with them is time consuming as both transformed and non-transformed plants have to be screened.

## 1.2 Thiol methyltransferase-encoding gene: A new selectable marker

A new selectable marker system was recently developed (Koonjul et al, unpublished), which uses a thiol methyltransferase (*TMT)* gene (Attieh et al., 2002) encoding an enzyme capable of detoxifying thiocyanate ions (Attieh et al., 2000).

### *1.2.1 Thiol methyltransferase (TMT): Historical view*

Thiol methyltransferases (TMTs) are enzymes mostly found in *Brassicaceae* and several other plant families of the order Capparales. They are able to transfer methyl group of *S*-adenosyl-L-methionine (AdoMet) to thiol groups, including highly reactive thiocyanate ($^-$SCN) and bisulfide ($HS^-$) ions, as well as to halide ions, producing the corresponding methyl compounds that are often volatile (Attieh et al., 1995; Attieh et al., 2000).

When originally discovered, TMT was thought to be a halide/bisulfide methyltransferase (H/BMT) (Attieh et al., 1995). Its function was initially thought to be the methylation of the halide ($Cl^-$, $Br^-$, and $I^-$) or bisulfide ($HS^-$) ions. It was shown that the methylating activities for both halides and bisulfide co-purified at about the same ratio, an indication that both methylation reactions might be catalyzed by the same enzyme. This possibility was confirmed by kinetic analysis that showed competitive inhibition between $I^-$ and $HS^-$. These results demonstrated that the halide and bisulfide methylation was carried out by the same enzyme, the halide/bisulfide methyltransferase (H/BMT), and both substrates bind to the same active site of the protein.

### *1.2.1.1 The role of TMT*

Because of its methylation activity, TMT was suggested to play a role in the detoxification of its phytotoxic substrates. The first suggestion was that the enzyme might be involved in detoxifying $Cl^-$ as a part of plant's salt-tolerance mechanism. However, the enzyme activity was very low in halophytic plants, and was not affected by plant salinization, providing no evidence for a role in salt tolerance (Saini et al., 1995).

Since the enzyme activity was highest in species of the sulfur-rich family Brassicaceae, which is known to produce $HS^-$, one of the enzyme's substrates, another suggestion was that TMT might be involved in sulfur metabolism. Several potential substrates were assayed based on the ability of various compounds to compete with $I^-$ for the enzyme's active site and thus inhibit $I^-$ methylation (Attieh et al., 2000). Among a large number of compounds screened, thiol compounds, especially $^-SCN$, thiophenol, 4,4'-thiobisbenzenethiol, and thiosalicylic acid were highly inhibitory of $I^-$ methylation, suggesting that they might be methylated by TMT. This suggestion was confirmed with a radiometric assay, in which radiolabelled methylated products were detected when their corresponding substrates, the pure TMT, and the labeled methyl donor AdoMet were present in the medium (Attieh et al., 2000).

These TMT substrates or their analogues are known to arise from the hydrolysis of the secondary metabolites glucosinolates (Attieh et al., 2000). Glucosinolates are natural organic compounds found in all plants belonging to the order Capparales (Duncan, 1991). They are glucosides characterized by a

thioglucose moiety, a sulfonated oxime, and a side chain derived from amino acids. Glucosinolates have various biological functions, including anticarcinogenic properties, effect on taste and flavor of food, and plant defense against insects, fungi, and herbivores (Jezek et al., 1999; Mikkelsen et al., 2002). Upon herbivore attack, these compounds are degraded by myrosinase, an endogenous thioglucosidase, into various toxic compounds (Halkier and Du, 1997) that are not only harmful to the attacking pathogens, but could be toxic for the plant itself. Among all these compounds, $^{-}SCN$ was found to be the most preferred substrate for the TMT (Attieh et al., 2000).

These results suggest that this methyltransferase could be involved in the methylation and hence detoxification of toxic thiol products of the glucosinolate pathway. TMT was also found to be associated with glucosinolates, as well as myrosinase, in the different plant parts, especially in younger tissues (Attieh et al., 2000), which further supports the finding that TMT is associated with the detoxification of the glucosinolate hydrolysis products.

#### *1.2.1.2 TMT isoforms*

TMT was purified to near homogeneity from *Brassica oleracea* L. (red cabbage) through five purification steps, and proteins from each step were separated on an SDS-PAGE (Attieh et al., 1995). The purification with HPLC on Protein Pak Q anion exchange column resulted in multiple peaks of the activity that were all able to methylate both $I^{-}$ and $HS^{-}$, suggesting the existence of multiple isoforms of the enzyme. Five isoforms, having molecular masses

between 26 and 31 kDa, were isolated (Attieh et al., 2000). All isoforms were shown to methylate different thiol products, including $^{-}SCN$ and $HS^{-}$, as well as the halide $I^{-}$.

### *1.2.1.3 TMT-encoding genes*

This class of newly identified enzymes, found to have at least five isoforms with different properties and molecular weights, was most likely derived from more than one gene. *Attieh et al. (2002)* isolated two cabbage cDNAs, *cTMT1* and *cTMT2*, that coded for TMT. The molecular characterization of the cDNAs cloned showed that *cTMT1* was 910 bp long encoding a 25.13-KDa protein, with pI 4.72. *cTMT2* was 875 bp long encoding a 25.02-KDa protein, with pI 4.73. The proteins encoded had the same length but the difference in the molecular masses was due to difference in 13 amino acids. These two cDNAs contained the conserved motifs I, II, and III of methyltransferases. Motifs I and III were shown to be present in N- and O- methyltransferases as part of the AdoMet binding site. Motif II was also widely found in methyltransferases but was not associated with active parts of the gene (Ni and Hager, 1998). On the other hand, these cDNAs did not share significant homology with other methyltransferase cDNAs. Besides, the protein translated did not show activities related to N-, O-, or S- methyltransferases. These findings suggested that TMT were distinct from other methyltransferases and hence they were considered novel plant enzymes that were unique, with unique classes of genes.

*1.2.2 Development of the selectable marker*

The idea of using *TMT* gene as a selectable marker was based on the notion that TMT-catalyzed conversion of $^-$SCN to volatile $CH_3SCN$ was a detoxification reaction (Attieh et al., 2000). This idea was supported by the findings (Koonjul et al, unpublished) that red cabbage (*Brassica oleracea*), which naturally contains *TMT*, was much more tolerant to $^-$SCN than tobacco, which does not contain the gene. Moreover, engineering *TMT* into tobacco (*Nicotiana tabacum*) plants increased their tolerance to $^-$SCN, and knocking a homologue of the gene out of *Arabidopsis thaliana* through T-DNA insertion reduced its tolerance to the ion. Taken altogether, these results suggested that *TMT* gene could be used as a selectable marker for plant transformation, such that if the *TMT* and a gene of interest were co-introduced into a population, only the individuals carrying *TMT* gene would survive in the presence of $^-$SCN while those lacking the gene would die or lag in growth.

The efficiency of this marker was successfully demonstrated in root cultures of eight dicot plants, all of which lacked the *TMT* gene, as well as for the selection of whole plants in three different species: *A. thaliana,* a dicot that contains a homologue of the gene (Attieh et al., 2002), *N. tabacum* (tobacco), a *TMT*-lacking dicot, and *Oryza sativa* (rice), a monocot that lacks the gene (Koonjul et al, unpublished). The details are summarized below.

Root cultures of eight dicots were transfected with *Agrobacterium rhizogenes* containing a vector with or without the *TMT* gene and the roots were grown on $^-$SCN-containing medium at a concentration inhibitory to the growth of

non-transformed roots. The roots that grew faster in the presence of $^{-}$SCN were selected as putatively transformed. PCR and TMT enzyme assay confirmed that in seven of eight species, all visually selected clones were TMT-positive. Thus *TMT*-based selection in these species was 100% accurate. The accuracy of selection in the eighth species was 75%.

Having thus established the proof of concept, the efficacy of *TMT* was tested in plant transformation. *A. thaliana*, tobacco, and rice plants were transformed with *A. tumefaciens* containing a vector having both *TMT* and *nptII* sequences. The use of these two genes together allowed the comparison between the efficacy of *nptII* and *TMT* as selectable markers. In the case of tobacco, 2.5 mM $^{-}$SCN inhibited the growth of wild type plants. This concentration of the ion was added to the regenerating calli. The plants that grew normally were selected and all of them were shown to contain the *nptII*, suggesting that *TMT* has provided 100% selection efficiency. When plants were grown on kanamycin-containing medium, only 80% of the selected plants contained the *TMT* gene, suggesting a less accuracy for selection with *nptII*. These results were similar for *A. thaliana* transformation. *Arabidopsis* plants were transformed using the floral dip method (Clough and Bent, 1998). Seedlings were grown on selection medium either containing $^{-}$SCN or kanamycin. When *TMT* was used as the selectable marker, 75% of the visually selected plants were proven to contain *nptII*. In contrast, when *nptII* was the marker for selection, only 58% of the selected plants actually contained the *TMT* gene. These results showed that in both dicot plants, selection with *TMT* was more accurate than the current most commonly used

marker, *nptII*. In the case of monocots, rice was used for transformation. In general, monocot transformation has a low yield of transformed plants. Only two plants were selected visually to be transformed and both plants contained *nptII*, suggesting 100% selection efficiency with *TMT*. However, no plants were regenerated when *nptII* was used for selection.

The above results showed that *TMT* could be used as an efficient selectable marker in conjunction with $^{-}$SCN, regardless of whether the plant itself does or does not naturally contain the *TMT* gene. As the *TMT* gene is found naturally in a number of plants (Saini et al., 1995), this marker bypasses the concerns over the risk of lateral transfer of foreign genes, such as antibiotic-resistance genes (Stewart et al., 2000; Miki and McHugh, 2004). The use of these genes as selectable markers has been criticized because of the potential for their transfer from transformed plants to microorganisms, which could lead to the development of resistance to these antibiotics in pathogenic microbes (Bertolla et al., 2000; Chiter et al., 2000). Although gene transfer occurred from plants to prokaryotes during evolution (Heinemann, 1991), the transfer was limited among distant organisms and was affected by various environmental factors, providing no evidence of the possibility of increasing the resistance of bacteria (Bertolla et al., 2000). However, critics argue that the risk, even if small, could be avoided with alternatives markers (Miki and McHugh, 2004). One such alternative is the *TMT* gene. It also has an additional advantage that the selective agent $^{-}$SCN is a natural less expensive compound that can be easily added to the medium.

## 1.3 Selectable markers: Avoiding side effects

To overcome any side effect of the marker genes after the transformation, the selectable markers are sometimes excised from the transgenic plants (Hare and Chua, 2002). Cre recombinase has been used to eliminate the selectable marker (Dale and Ow, 1991; Zhang et al., 2003; Wang et al., 2005; Cuellar et al., 2006), leaving the inserted gene of interest intact in the plant. This excision could avoid the concerns about genetically modified crops and facilitate the commercialization of these transgenic plants.

Alternatively, development of an inducible selectable marker would have the advantage of avoiding the need to excise the marker from the transgenic plants. In this case, the marker would be induced only during the selection process and then switched off once the selection has been accomplished, thus avoiding any effect of the marker gene on the growth and development of selected transgenic plants. To our knowledge, no inducible selectable marker is currently available.

## 1.4 Development of *TMT* as an inducible selectable marker

Development of *TMT* into an inducible marker could be possible if a suitable promoter that turns on the gene only during the selection could be found. The object of this work is to find such a promoter. Ideally, this should involve the regulation of the *TMT* gene by one of the TMT's substrates, such as $^{-}$SCN, that is used as a selective agent. In general, gene expression is regulated by promoters found upstream of a gene's transcription site. Promoters are themselves regulated by a variety of factors such as heat (Holtorf et al., 1995), cold (Mujacic et al.,

1999), pathogen attack (Kooshki et al., 2003), wounding (Keil et al., 1990) and chemicals (Yurimoto et al., 2000). The identification and isolation of a promoter that is positively regulated by the ion $^{-}$SCN, would require three steps:

1. Identification of genes expressed only in the presence of $^{-}$SCN.
2. Isolation of the upstream promoters regulating these genes.
3. Test the promoter by transformation to prove its inducibility by $^{-}$SCN.

The first step was done by comparing gene expression in untreated versus $^{-}$SCN-treated plants. Two plants were used in this study, rice and *A. thaliana*, since their full genome is already sequenced and it would be convenient to find the complete sequence of the $^{-}$SCN-responsive gene as well as the upstream promoter sequence. The comparison of gene expression was done through differential display and microarray analysis. The genes that were found to be up-regulated in the presence of $^{-}$SCN were isolated and identified. The aim was to identify the promoters regulating these genes, i.e. the $^{-}$SCN-induced promoters. The second step then involved the isolation of the upstream sequence of the genes, containing the promoter region, by polymerase chain reaction. In the third step, this region was tested for its ability to induce gene expression only in the presence of $^{-}$SCN. The test was done by introducing the sequence in a binary vector upstream of a reporter gene, *gusA*, so that the putative $^{-}$SCN–induced promoter regulates the expression of *gusA*. The construct was transformed into *A. rhizogenes*. Transformation of plants with *A. rhizogenes* leads fast-growing roots (Visser et al., 1989; Rivoal and Hanson, 1994). It would be convenient to initially test the promoter activity in cultured roots for rapid results. Besides, *A.*

*tumefaciens*-mediated transformation for which the floral dip of *A. thaliana* was used to test the promoter activity in the whole plant (Zhang et al., 2006). Transgenic plants that would integrate the chimeric gene and grow normally in the selective medium would be selected and tested for *gusA* expression. Some of these plants would be exposed to $^{-}$SCN, and others used as a control.

Following exposure to $^{-}$SCN, the promoter would be activated and would induce the expression of *gusA* that encodes the enzyme beta-glucuronidase (GUS). GUS is able to cleave a chromogenic substrate, 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), resulting in a blue color in the plant cells where *gusA* is expressed. This assay is widely used to test promoter activity since it is inexpensive and easy to perform and visualize (Jefferson et al., 1987). In the absence of $^{-}$SCN, *gusA* expression would not be induced, and plant cells would not stain blue in the presence of X-Gluc. To test the promoter activity, *gusA* expression would be tested by incubating the transgenic plant tissues with X-Gluc and observed for possible blue staining.

# 2. MATERIALS AND METHODS

## 2.1 Plant materials

Two plant species were used: *Arabidopsis thaliana* Columbia-0 ecotype and *Oryza sativa* (rice) Japonica and Indica types. The seeds of both species were surface sterilized as follows: For rice seeds, sterilization involved a wash with 70% ethanol for 1 minute, followed by a 15-minute wash with 2.5% (v/v) NaClO containing 2 drops of tween-20, and three successive washes with sterile water for 5 minutes each. *Arabidopsis* seeds were washed with 50% ethanol for 1 minute, followed by a wash with 0.2% (v/v) SDS/50% (v/v) NaClO for 10 minutes, and three successive washes with sterile water for 10 minutes each. Sterile seeds were grown on Murashige and Skoog (MS) medium supplemented with different $^{-}$SCN concentrations.

## 2.2 Optimization of $^{-}$SCN concentrations

*A. thaliana* naturally contains the *TMT* gene. In order to study genes induced by $^{-}$SCN, the plants were exposed to a sublethal concentration of the ion; a concentration at which the TMT enzyme is no longer able to methylate all of its substrates but the plant is still able to survive. *Arabidopsis* seeds were germinated and grown on MS medium with different $^{-}$SCN concentrations, ranging from 0.25 mM to 100 mM to find the optimum ion concentration. Rice, which does not contain the *TMT* gene, is extremely sensitive to $^{-}$SCN. Hence, rice seeds were germinated on basal MS media then transferred to grow on MS media containing

different concentrations of $^{-}$SCN, ranging from 5 to 100 mM, for a period of one to three days.

## 2.3 RNA extraction

RNA was extracted from control plants and those exposed to different concentrations of $^{-}$SCN. In the case of rice, the two-week old plants exposed to different $^{-}$SCN concentrations for different days were ground to a fine powder in liquid nitrogen, and RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Missisauga, ON) according to manufacturer's recommendations. For *Arabidopsis,* plants were about three weeks old. The whole plants were ground in liquid nitrogen and 1 ml of Trizol reagent was added to 100 mg of powder. The mixture was incubated at room temperature for 5 minutes to break down the cells then 0.2 ml chloroform was added and the solution was incubated for 15 minutes at room temperature, followed by a centrifugation in a microcentrifuge at 4°C at 12,000g for 15 minutes. The upper aqueous phase obtained was saved and mixed with 0.25 ml of isopropanol. The solution was again incubated at room temperature for 10 minutes and centrifuged for 10 minutes under the same centrifugation conditions. After centrifuging, the supernatant was removed and the RNA pellet was dissolved in 70% (v/v) ethanol solution, and centrifuged at 4°C at 7,500 g for 5 minutes. All the ethanol solution was aspired and the pure RNA pellet was resuspended in 30 µl RNase free-water. The RNA in the extracts was quantified by measuring their absorbance at 260 nm using a DU-65 spectrophotometer (Beckman Instruments, Mississauga, ON). The RNA purity

was determined by comparing absorbance at 260 nm and 280 nm; an A260/280 ratio of 1.8 to 2 indicated that the RNA was pure. RNA integrity was determined by electophoresing 1 μg of RNA on a 1% (w/v) agarose gel, staining it with ethidium bromide and observing the bands under UV light.

## 2.4 Microarray analysis

An Affymetrix GeneChip Arabidopsis ATH1 Genome Array was purchased from Genome Quebec and McGill Center (McGill University, Montreal, QC) and microarray analysis was carried out by the center's technicians. Control and $^{-}$SCN-exposed RNA were sent to be reverse transcribed and screened with the chips. Although multiple replicates for analysis of differentially expressed genes with microarray chips were recommended, only one replicate of each case was used, because of the high cost of each chip. Besides, it was considered that the different concentrations of $^{-}$SCN are the different replicates, however only one replicate for the control was used. Genes that showed an expression in treated samples with a fold change above 2.5 compared to the expression in the control samples were considered as up-regulated by $^{-}$SCN. These genes were isolated and tested by reverse transcription-polymerase chain reaction (RT-PCR) and northern blot analysis. These two tests were critically important to confirm the results obtained with the microarray analysis.

## 2.5 Reverse transcription polymerase chain reaction (RT-PCR)

Several genes that showed up-regulation in the presence of $^{-}$SCN in the microarray chips were tested by RT-PCR using the One-Step RT-PCR kit (QIAGEN, Missisauga, ON). Forward and reverse primers were designed for each gene. Two PCR reactions were prepared for each gene; one with control RNA, and the other with $^{-}$SCN-exposed RNA. Each reaction was prepared for a final volume of 50 µl and contained 1 µg of RNA, 0.6 mM of each primer, 1X enzyme buffer, 400 µM of each dNTP, and 2 µl of QIAGEN One-Step RT-PCR enzyme mix. The reaction mixture was subjected to the following steps in a thermal cycler: 30 minutes at 50°C for reverse transcription, 15 minutes at 95°C for enzyme activation, followed by 35 amplification cycles of 30 seconds at 94°C for cDNA denaturation, 30 seconds at 55°C for primer annealing, and 90 seconds at 72°C for elongation, and a final elongation at 72°C for 10 minutes. During the 35 amplification cycles, 4 µl of each PCR reaction was removed after every 5 cycles and loaded on an agarose gel to study the expression of the gene in the presence and absence of $^{-}$SCN. The intensity of the band on the agarose gel is an indication of the level of expression of the gene. Actin2 primers were used as a control to ensure equal amounts of RNA. Two PCR reactions were prepared using the actin2 primers; one with control RNA, and the other with $^{-}$SCN-treated RNA. The control PCR reactions was prepared as mentioned for the microarray-identified genes, and subjected to the same PCR conditions. The control PCR products were also loaded on the agarose gel, to see the actin gene that is

expected to show equal intensities in both cases, suggesting that equal amounts of control and $^{-}$SCN-treated RNA were used.

## 2.6 Northern blot analysis

### *2.6.1 RNA transfer*

Rice and *Arabidopsis* RNA from control and $^{-}$SCN-treated plants were loaded on 1% (w/v) agarose gel for 1.5 hours at 220 volts in 1X MOPS buffer (0.5 M MOPS pH 7.3 and 0.01 M EDTA). Ten µg of each RNA sample previously treated with 3 M sodium acetate (NaOAc) and 100% (v/v) ethanol was centrifuged in a microcentrifuge at 4°C at 12,000 g for 30 minutes and further purified by suspension of the RNA pellet with 500 µl of 70% (v/v) ethanol. The RNA was microcentrifuged at 4°C at 12,000g for 10 minutes and dried in Savant SpeedVac plus model SC110A to remove all residual ethanol. After all the ethanol was aspired, the RNA pellet was then re-suspended in 4.8 µl buffer A (3X MOPS buffer) and 9.2 µl F/F solution (9% (v/v) formaldehyde and 73% (v/v) formamide). The mixture was incubated at 70°C for 10 minutes for denaturation of the secondary structure. The different samples were loaded on the gel after addition of the loading dye (322 µl buffer A, 5 mg cyanol xylene, 5 mg bromophenol blue, 400 mg sucrose, 178 µl of 37% (v/v) formaldehyde, and 500 µl deionized formamide), stained with ethidium bromide and visualized under UV light. The visualization under UV light allowed the verification of the integrity and the quantity of the RNA. These RNAs were then transferred to a positively charged nylon transfer membrane (Fisher, Ottawa, ON). The agarose

gel was placed with the entry of wells faced down on a large Whatman paper that had its extremities soaked in 20X SSC solution (3 M sodium chloride and 0.3 M sodium citrate, pH 7). The nylon membrane was soaked in 20X SSC solution and placed between the gel and three presoaked and one dry Whatman papers. The assembly was covered with Saran wrap and a layer of paper towels, a glass plate and a heavy book were placed on top. After 16 hours, the membrane was removed and the RNAs were fixed on the membrane through UV cross-linking.

*2.6.2 Probe preparation and hybridization*

Probes were prepared from different candidate genes identified by microarray analysis using the Strip EZ DNA Kit (Ambion, Austin, TX), according to the manufacturer's recommendations. The cDNAs were radioactively labeled with fresh $\alpha$-$P^{32}$ labeled dATP (Perkin-Elmer, Massachusetts, USA). Before hybridization with the probes, the membranes containing the transferred RNA were pre-hybridized with 50% formaldehyde buffer (50% (v/v) deionized formamide, 30% (v/v) of 20X SSC, 10% (v/v) of 50 X Denhardt's solution, 5% (v/v) of 20% (v/v) SDS, 4% water, and 1% (v/v) of 10 mg $ml^{-1}$ salmon sperm DNA) at 45°C for 2 hours. The probes were purified using the quick spin G50 Sephadex columns, denatured and added to fresh 50% formaldehyde buffer. The incorporation of radioactive dATP was assayed in 1 µl of the probe using a liquid scintillation counter. The membranes were incubated with the radioactive probes at 45°C for 12 hours.

*2.6.3 Washes, exposure and development of membranes*

Following hybridization, the membranes were washed with different SSC solutions. The washing started in a solution of 2x (v/v) SSC, 0.1% (v/v) SDS at 35°C for 30 minutes. The wash was repeated twice, followed by 2 more washes each in a mixture of 1X (v/v) SSC and 0.1% (v/v) SDS at 45°C for 30 minutes. The final wash was done once in a 0.1% (v/v) SSC and 0.1% (v/v) SDS solution at 50°C for 10 minutes. The membrane radioactivity was monitored by a Geiger counter. The membranes were exposed to the films for 12 to 24 hours, and the films were developed in the automatic developer Curix 60 (Agfa, Pointe Claire, QC). Some membranes were exposed in cassettes for 12 hours and the films were developed in the phosphorimager Typhoon 9200 (Amersham Biosciences, Piscataway, NJ, USA) for faster results.

## 2.7 Differential display

The RNAimage Kit (GenHunter, Nashville, TN) was used for the analysis of genes differentially expressed between $^{-}$SCN-treated and control rice and *Arabidopsis* plants, using the protocol of Liang and Pardee (Liang and Pardee, 1992). For rice, cDNA from plants exposed to 0, 10, and 50 mM $^{-}$SCN for 1 day were studied using the three anchored primers (H-$T_{11}$A, H-$T_{11}$C, and H-$T_{11}$G) combined with the arbitrary primers HAP1 to HAP8 (Liang et al., 1994). *Arabidopsis* cDNA was from plants exposed to 0, 1, and 2 mM $^{-}$SCN during germination and early seedling growth. The three anchored primers were also used in combination with arbitrary primers HAP9 to HAP16. The Choice of

different primer combinations was random. The cDNAs were radioactively labeled with fresh $\alpha$-$P^{33}$ labeled dATP (Perkin-Elmer, Massachusetts, USA). The 6% denaturing gel was prepared as follows: 42 g of urea, 5.7 g of acrylamide, 0.3 g of bis-acrylamide, 20 ml of 5X TBE buffer (54 g Tris base, 27.5 g boric acid, and 0.01 M EDTA pH 8.0) were dissolved in 40 ml water. The solution was mixed, filtered using a 0.45 micron filter, and degassed. Just before loading the gel, 500 µl of freshly made 10% (w/v) ammonium persulfate and 50 µl of TEMED were added to the solution for polymerization. The samples were loaded and the gel was electrophoresed for 4 hours at constant 60 W, with a voltage of 3500 V and a current of 150 mA.

**2.8 Sequencing and sequence analysis**

Bands from the gels showing putative differentially expressed genes in both rice and *Arabidopsis* were extracted, purified, amplified, and sequenced using the appropriate anchored and arbitrary primer combinations used in the differential display analysis. The sequencing was done using the University Core DNA and Protein Services, University of Calgary, AB. Each primer (3.2 µM) was mixed with 100 ng of cDNA and sent for sequencing at an annealing temperature of 55°C. Sequences were analyzed, corrected and subjected to BLAST analysis in GenBank to identify the corresponding gene sequences.

## 2.9 Slot blot analysis

Equal amounts of cDNAs amplified from the candidate differential display bands were loaded on a positively charged Slot Blot membrane. The membrane was dried and UV cross-linked to fix the DNA. Probes were prepared from 3 μg RNA from control and $^{-}$SCN-exposed plants, 25 mM of each dGTP, dCTP, and dTTP, MMLV enzyme, 1X RT buffer, and 10 mM of the primers H-$T_{11}$A, H-$T_{11}$C, and H-$T_{11}$G. The probes were radioactively labeled with fresh α-$P^{32}$ labeled dATP (Perkin-Elmer, Massachusetts, USA). The solutions were incubated at 65°C for 5 minutes to denature secondary structures of RNA, followed by 60 minutes at 37°C for reverse transcription. The MMLV enzyme was added after 10 minutes at 37°C. The reactions were stopped by incubation for 5 minutes at 75 °C.

The membranes were pre-hybridized with 50% formaldehyde. The probes were purified, denatured, added to 50% formaldehyde buffer and hybridized for 12 hours with the membrane containing the different cDNAs. After hybridization, the probes were removed and the membranes were subjected to a series of washes with different SSC solutions of increasing stringency as described in section 2.6.3, and exposed for 12 to 16 hours to a film for development.

## 2.10 Northern blot analysis

Rice or *Arabidopsis* RNA from control and $^{-}$SCN-treated plants was loaded on a 1% agarose gel, transferred to a nylon membrane and cross-linked as described in section 2.6.1. Probes were prepared from the different candidate

bands amplified from the differential display gel following the same procedure for the preparation of the probes for microarray-identified genes. After pre-hybridization and hybridization of the northern membranes with the different probes, the membranes were washed with the different SSC solutions, and exposed to films for 12 to 24 hours to be developed.

**2.11 Promoter isolation**

Since the genome of both rice and *Arabidopsis* are fully sequenced, genes that were found differentially expressed by $^{-}$SCN through differential display analysis and northern blotting were sequenced and characterized. The upstream sequence of each gene was isolated and amplified through polymerase chain reaction (PCR). For rice, a 2067 bp sequence upstream of the gene (rice upstream sequence-RU) was amplified by PCR after several rounds of optimization using the two primers: forward primer RUF 5'-GAGAGGATCCGGCATATACACCGTCCATCC-3' and reverse primer RUR 5' GAGACCATGGGCTGGCCACTGGTATGACTT-3'. The forward and reverse primer sequences started with *Bam*HI and *Nco*I restriction sites, respectively, to facilitate ligation of the upstream sequence with a promoterless vector containing the GUS reporter gene. One hundred ng of genomic rice DNA, 2.5 μM of each primer, 200 μM of each dNTP, 1 U of Taq polymerase and 1X of the corresponding enzyme buffer (QIAGEN, Mississauga, ON) were mixed in a 40 μl solution. The PCR program included an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles of denaturation (94°C for 1 minute), primer

annealing (60°C for 1 minute) and elongation (72°C for 3 minutes). The amplification ended with a final elongation at 72°C for 10 minutes.

In the case of *Arabidopsis*, a 502 bp upstream region of the gene (*Arabidopsis* upstream sequence- AU) was amplified by PCR using the forward primer AUF with *Bam*HI restriction site 5'-GAGAGGATCCGTCTGGCTCAACGGTAACT-3' and the reverse primer AUR with *Nco*I restriction site 5'-GAGACCATGGGGATTCGGTTTCTGGAGGAT-3'. The PCR program was the same as that for rice, except that the elongation was for 1 minute.

**2.12 Promoter analysis**

RU, AU, and pCambia 1291Z were cut with *Nco*I and *Bam*HI, using 500 ng of each DNA and 10 units of each restriction enzyme (Startagene, Texas, USA) with 1X corresponding buffer at 37°C for 12 hours. The DNA and the vector with sticky ends were loaded on a 1% agarose gel and the corresponding bands were cut from the gel using a clean sharp scalpel. The bands were purified using the Qiagen Gel Extraction Kit (QIAGEN, Mississauga, ON) according to the manufacturer's recommendations.

Following purification, RU and AU were each ligated with pCambia, using 0.4 U of T4 DNA ligase (Invitrogen, Burlington, ON), and a vector to insert ratio of 1:5, with 1X of the corresponding ligase buffer in a 20 µl reaction, and incubated at room temperature for 4 hours.

## 2.13 Preparation of electrocompetent bacteria

Different electrocompetent bacteria were prepared; *E. coli* XL-1 Blue MRF', *Agrobacterium rhizogenes* A4, and *A. tumafeciens* LBA 4404. For *E. coli,* a single colony was isolated, inoculated in 5 ml LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride, and a pH of 7.0) with 4 μg $ml^{-1}$ tetracycline (the strain is resistant to tetracycline), and incubated at 37° C for 16 hours. The culture was transferred to 500 ml LB medium with tetracycline and grown at 37° C with vigorous shaking until it reached an $OD_{600}$ of 0.5. The culture was chilled on ice and then centrifuged to pellet the bacteria. The pellet was resuspended in 10% cold glycerol and centrifuged several times until most of the salt is washed out. The electrocompetent cells were stored at -80° C.

For both *Agorbacterium* strains, the same protocol was used except that the incubation temperature was always at 28°C, and the medium used for growth was MYA (0.5% (w/v) yeast extract, 0.05% (w/v) casamino acids, 0.8% (w/v) mannitol, 0.2% (w/v) ammonium sulfate, 0.5% (w/v) sodium chloride, and with a pH of 6.6). Besides, as *Agrobacterium is* not antibiotic resistant, the bacteria were initially grown on MYA plates and MYA medium without antibiotic.

## 2.14 Electroporation with *E. coli*

Each ligation reaction was transformed into electrocompetent *E.coli* through electroporation using a cell porator (GIBCO BRL, Invitrogen, Burlington, ON). Bacteria (23 μl) were thawed on ice and mixed with 2 μl of ligation reaction. The 25 μl reaction was introduced between the electrodes of the

electroporation cuvette and electroporated under a capacitance of 330 μf, a fast charge rate, and a low resistance. Immediately after electroporation, 1 ml of LB medium was added to the cuvette, the mixture was transferred to a new tube, and incubated at 37°C for 1 hour with shaking. Fifty and hundred μl of the transformed bacteria were plated on LB agar plates with chloramphenicol, and the plates were incubated at 37°C overnight. Chloramphenicol was used for the selection of colonies containing the vector pCambia 1291Z at a final concentration of 20 μg $ml^{-1}$. Five to ten colonies grown on each plate were picked, inoculated in 5 ml LB medium with chloramphenicol, and incubated at 37°C for 18 hours with vigorous shaking. A 3 ml aliquot was used for plasmid extraction using the Qiaquick miniprep extraction kit (QIAGEN, Mississauga, ON), following the manufacturer's recommendations. The plasmid was quantified and 100 ng was amplified by PCR using the forward and reverse primers for the inserted upstream sequence, i.e. primers RUF and RUR for rice insert and primers AUF and AUR for *Arabidopsis* insert. The PCR products were analyzed on a 1% (w/v) agarose gel to confirm the presence of the insert within the pCambia vector. The remaining 2 ml were preserved in a glycerol solution at -80°C for future use.

The plasmid obtained by plasmid miniprep was transformed into both electrocompetent *A. rhizogenes* and *A. tumefaciens* by electroporation. The same protocol was repeated, except for the incubation temperature (28°C instead of 37°C), the medium, and agar plates used (MYA instead of LB). Again, colonies containing the appropriate plasmid were stocked in a glycerol solution at -80°C.

**2.15 Tobacco root transformation**

Tobacco plants were grown for 45 days. Ten plants were used from two different tobacco types, Xanthi and Zimmer. Each plant was defoliated and stems were cut, washed thoroughly, and then sterilized with sterile bleach solution and two drops of Tween-20, followed by three washes with sterile water. Sterilized stems were then cut into 4 cm sections and placed upside down in MS media. Each stem was infected immediately with *A. rhizogenes* containing the construct of pCambia1291Z-UpstreamSequence and incubated at 23°C under continuous light (Visser et al., 1989; Rivoal and Hanson, 1994). Roots started to emerge from the stem segments after ten days of incubation. They were left to grow for about five more days and then cut out root by root and transferred to MS media with 0.2% phytagel and 500 $\mu g\ ml^{-1}$ carbenicillin. Roots were left to grow for a week in darkness, and newly grown parts were transferred to new MS media with 250 $\mu g\ ml^{-1}$ carbenicillin, then to 100 $\mu g\ ml^{-1}$, 50 $\mu g\ ml^{-1}$ carbenicillin and finally to MS media without carbenicillin. Carbenicillin is a bacteriostatin that progressively frees the medium of *A. rhizogenes* growing on the roots while maintaining root growth. For pCambia 1291Z, hygromycin was used as the selective antibiotic at a concentration of 25 $\mu g\ ml^{-1}$ for tobacco. The antibiotic was added with lower concentration of carbenicillin (50 $\mu g\ ml^{-1}$) and in MS media without carbenicillin. In the final medium, the roots that grew normally in the presence of the selective antibiotic were selected and transferred to new MS media with 25 $\mu g\ ml^{-1}$ chloramphenicol. Some of these roots were transferred to

MS media with 25 μg $ml^{-1}$ chloramphenicol and 1 mM $^{-}$SCN. The visually selected roots were grown for an additional two weeks to have enough materials to test them by GUS assay. This assay allowed the analysis of *gusA* expression in control roots (no $^{-}$SCN) and roots exposed to $^{-}$SCN.

*2.15.1 GUS Assay on tobacco roots*

Roots grown normally in hygromycin–supplemented media either containing or lacking $^{-}$SCN were harvested in 90% acetone. The samples were incubated for 20 minutes at room temperature, placed on ice, and washed in the staining buffer (50 mM sodium phosphate buffer pH 7.2, 0.2% (v/v) Triton X-100, 2mM potassium ferrocyanide, and 2 mM potassium ferricyanide). The staining buffer was removed and fresh buffer with 2 mM GUS reagent X-Gluc was added. The samples were infiltrated under vacuum for 20 minutes and left for 16 hours at room temperature in the dark for GUS reaction to take place. The solution was removed and the roots were washed with a 20-35-50% (v/v) ethanol series for 30 minutes each. The samples were then immersed in the fixative solution FAA (50% (v/v) ethanol, 10% (v/v) glacial acetic acid, and 5% (v/v) formaldehyde) for 30 minutes followed by a wash with 70% (v/v) ethanol. Tissues were left in the ethanol solution for 2 hours to remove all chlorophyll before examination under the dissecting microscope.

## 2.16 *Arabidopsis* mesophyll protoplasts transformation

The protocol of Sheen (Sheen, 2002) was used for transfection of protoplasts of *Arabidopsis* mesophylls with the plasmid DNA. Leaves were cut carefully and incubated in cellulose/macroenzyme solution for 3 to 4 hours until the protoplasts were released, turning the solution green. The solution was filtered through a 35 μm nylon mesh in a 30 ml centrifuge tube and centrifuged in Beckman GS15R centrifuge at 4°C at 100 g for 2 minutes to pellet the protoplasts. The pellet was washed and resuspended in W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 2 mM MES pH 5.7) to get $10^6$ protoplasts per ml. The solution was centrifuged again under the same centrifugation conditions and the protoplast pellet resuspended in MMg solution (0.4 M mannitol, 15 mM $MgCl_2$, and 4 mM MES pH 5.7) for PEG transfection. For each sample, 100 μl of protoplasts were added to 10 μl of the plasmid (2 μg) in microcentrifuge tube and mixed well. Twelve samples for the pCambia construct, three samples for positive control (Super GUS vector), and three samples for negative control (pCambia1291Z vector alone) were prepared. Then PEG solution (4 g PEG-4000, 200 mM mannitol, and 100 mM $CaCl_2$) was added and mixed with the protoplasts and DNA. The solution was incubated for 30 minutes, diluted in W5 solution, and centrifuged in a microcentrifuge at 4°C at 100 g for 1 minute to remove PEG. The protoplast pellet was resuspended in W5 solution and added to 1 ml W5 solution in 6-well tissue culture dishes. The dishes were left for 16 hours with shaking at room temperature.

*2.16.1 GUS assay on protoplasts*

Transformed protoplasts were transferred to microcentrifuge tubes and centrifuged in a microcentrifuge for 5 minutes at 1,200 rpm to pellet the protoplasts. The pellet was resuspended in GUS extraction buffer (50 mM $NaPO_4$ pH7, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% (w/v) sodium lauryl sarcosyl, and 0.1% (v/v) Triton X-100), and incubated on ice for 5 minutes. Each sample was subjected to 4 cycles of ultrasound to burst the protoplasts and centrifuged in a microcentrifuge for 5 minutes at 14,000 rpm. The supernatant containing the proteins was transferred to a new tube. Five µl of the supernatant was added to 245 µl of GUS reagent (4.4 mg $ml^{-1}$ MUG (4-methylumbelliferyl-β-D-glucuronide) in 20% (v/v) methanol, in GUS extraction buffer). At times 0, 60 and 180 minutes, a dilution of one-tenth of each sample in GUS stop-buffer (0.2 M $Na_2CO_3$) was quantified using the spectrophotometer fMax Fluorescence Microplate Reader, and at the excitation and emission wavelengths of 360 and 455 nm, respectively.

**2.17 Transformation through floral dip**

Two month old *Arabidopsis* plants were transformed using the floral dip method of Zhang *et al.* (Zhang et al., 2006). Positive clones of *A. tumafeciens* were plated on MYA medium with chloramphenicol. A single colony was inoculated into 5 ml MYA broth with chloramphenicol and grown at 28°C for two days. The five ml culture was then added to 500 ml MYA broth and incubated at 28°C until the cells reached the stationary phase at $OD_{600}$ of 1.5. The culture was

centrifuged in Beckman J2-HS centrifuge at 4,000 G for 10 minutes to pellet the bacteria. The pellet was resuspended in 5% (w/v) sucrose solution. Just before dipping, 0.02% (v/v) of Silwet L-77 was added to the solution, and six plants were subject to floral dip using the authors' recommendations. The plants were wrapped in Saran wrap and put on their sides for 16 hours, after which the Saran wrap cover was removed and the plants were transferred to a growth chamber and left to grow in long days (16-24 hours of light per day) for one month. The seeds were collected at maturity and sterilized, through a single wash with 70% ethanol containing 2 drops of Tween-20 for 5 minutes, followed by three washes with 95% ethanol for 5 minutes each. The seeds were spread on MS media containing 25 $\mu g\ ml^{-1}$ hygromycin and 100 $\mu g\ ml^{-1}$ carbenicillin, placed at 4°C for three days for vernalization then transferred to grow in a growth chamber at 23 °C under continuous light (50-100 microEinsteins) for 10 days. The plants showing normal growth were transferred for another 10 days to fresh MS media with the same antibiotic. The experiment was stopped at this point because there was no time to continue and wait for the selected plants to grow for another month. GUS assay on transgenic plants could be done as in section 2.17.1.

# 3. Results

## 3.1 Optimization of $^{-}$SCN concentrations

The first step in the identification of $^{-}$SCN-induced promoters was to expose *A. thaliana* and rice seeds or plants to the optimal concentrations of the ion.

For *A. thaliana*, it was shown that seeds could germinate and grow at $^{-}$SCN concentrations below 2 mM. Figure 1 shows the growth of plants under different concentrations of the toxic ion. At 0.5 mM (Figure 1b), the seeds germinated and grew normally compared to those grown on normal MS media (Figure 1a). At 1 mM $^{-}$SCN (Figure 1c), the plantlets showed reduction in growth. Plants in normal media were healthy and green, whereas plants in media containing 1 mM $^{-}$SCN were shorter in length, and had a lighter green color. This result indicated that the $^{-}$SCN concentration had an effect on the phenotype of the plants, implicating a possible effect on the gene expression. At 2 mM $^{-}$SCN (Figure 1d), the effect of $^{-}$SCN was more visible. At concentrations above 2 mM (4 mM, Figure 1e), a low percentage of seeds germinated and the growth of the plants were drastically reduced, indicating that $^{-}$SCN started to be toxic for the plants. As shown in Figure 1f, at 10 mM $^{-}$SCN, the seeds were merely germinating and plant growth was drastically reduced. Figure 1g represents a comparison in the growth of a plant in normal medium, at 0.5 mM, and 1 mM $^{-}$SCN. The same pattern of growth was observed for control and 0.5 mM $^{-}$SCN-treated plant. The difference in growth was observed when the plant was exposed to 1 mM $^{-}$SCN.

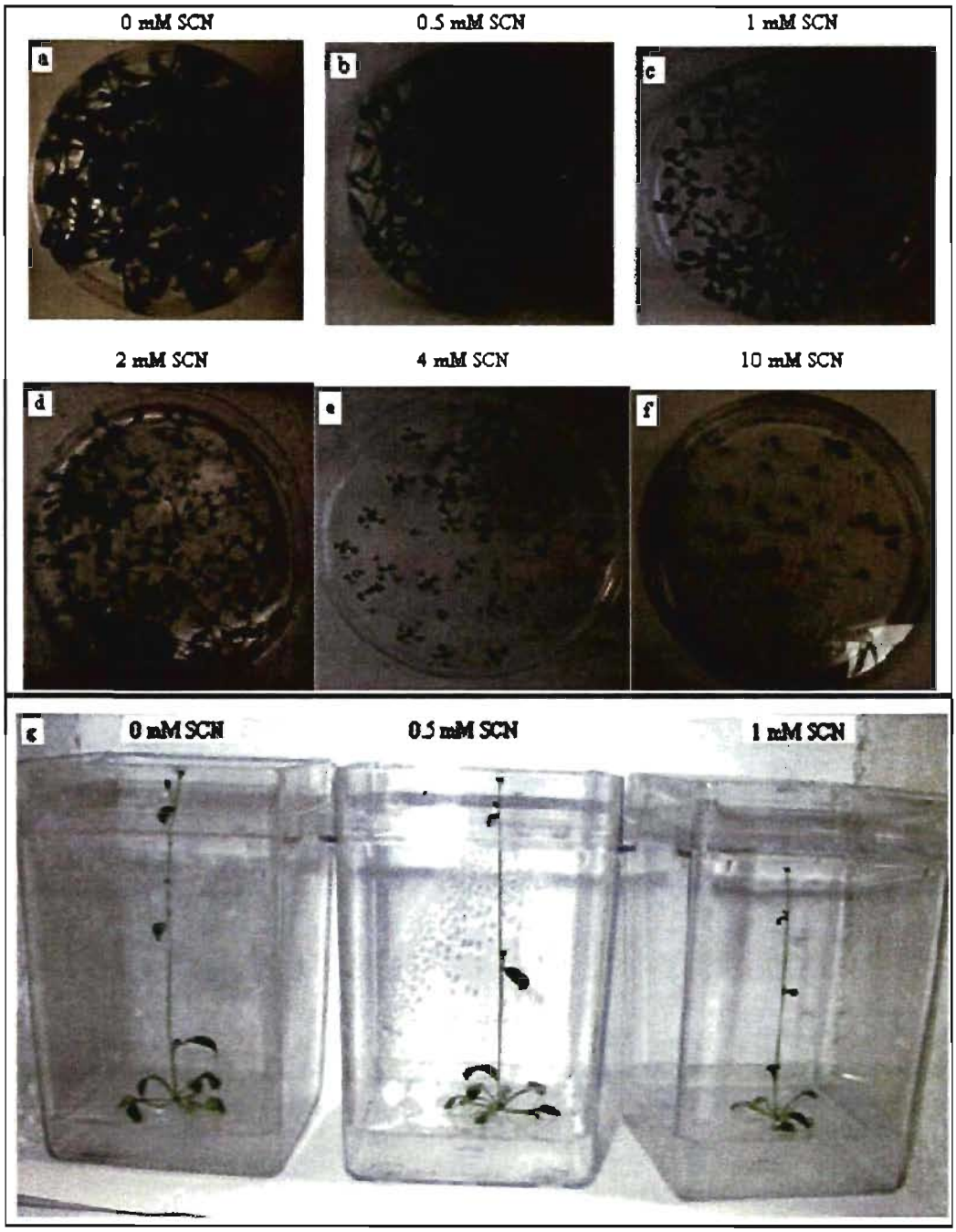
0 mM SCN
0.5 mM SCN
1 mM SCN
a
b
c
2 mM SCN
4 mM SCN
10 mM SCN
d
e
f
g
0 mM SCN
0.5 mM SCN
1 mM SCN

Figure 1. Response of *A. thaliana* plants to different concentrations of $^{-}$SCN. Plantlets growing in (a) unsupplemented MS medium, (b) under 0.5 mM $^{-}$SCN, (c) 1 mM $^{-}$SCN, (d) 2 mM $^{-}$SCN (e) 4 mM $^{-}$SCN, and (f) 10 mM $^{-}$SCN-containing media. (g) Comparison of plants growing in normal, under 0.5 mM, and 1 mM $^{-}$SCN-containing media.

As mentioned in section 2.2, rice plants do not contain the *TMT* gene. In contrast to *A. thaliana*, seeds were not able to germinate in media containing $^{-}$SCN, due to the ion toxicity. Therefore, seeds were germinated in unsupplemented MS medium, and seedlings were transferred to $^{-}$SCN-containing media. Plants were able to grow in the media containing less than 50 mM $^{-}$SCN (5 mM, 10 mM, 25 mM, and 50 mM). Plants were dead above this concentration (figures not shown). Pants were showing reduced growth in concentrations below 50 mM $^{-}$SCN, compared to those grown in normal MS media, but they were able to survive. Note that although *A. thnaliana* contains the *TMT* gene, they were only able to grow at lower concentrations of In terms of time, the plants exposed to these $^{-}$SCN concentrations were left to grow for one, two or three days. After 4 days, plants were starting to die, indicating that they were not able to withstand the stress of the ion toxicity anymore. Rice was able to grow at a much higher concentrations than *A. thaliana*, because rice seeds did not germinate in presence of $^{-}$SCN. *A. thaliana* seeds germinated in the presence of the toxic ion, and were not able to withstand a higher concentration after germination and when they were growing.

After exposing plants to different $^{-}$SCN concentrations, it was concluded that $^{-}$SCN had an effect on *A. thaliana* plants at concentrations of 1 mM and 2 mM. Seeds would germinate and grow under these concentrations and plants would be used for analysis. In the case of rice, 1 mM, 5 mM, 10 mM, and 50 mM $^{-}$SCN-exposed plants for one day were chosen for analysis.

Total RNA was extracted from both control and $^{-}$SCN-exposed rice and *A. thaliana* plants. The amount of RNA isolated from a 100 mg plant material ranged from 100 ng to 2 μg. Figure 2 shows typical RNA samples from both plants at different $^{-}$SCN concentrations.

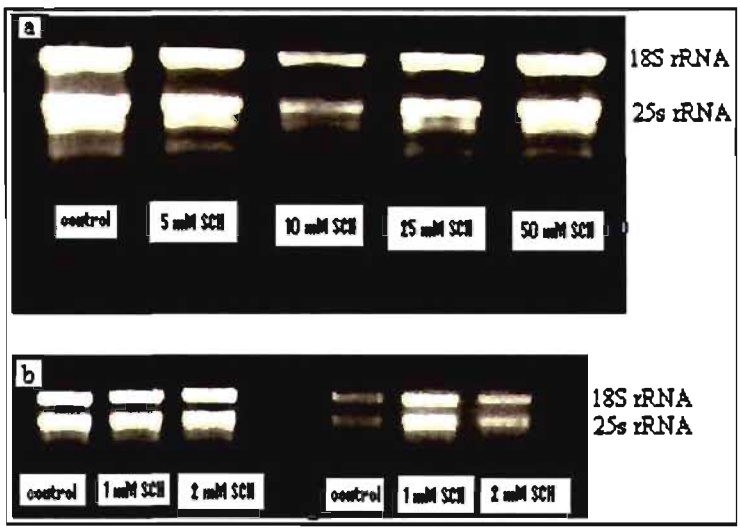


Figure 2. Checking RNA integrity on agarose gel. (a) Rice RNA extracted from control and $^{-}$SCN-exposed plants. (b) *A. thaliana* RNA extracted from control and $^{-}$SCN-exposed plants. The RNA that are not degraded are chosen for use in microarray and differential display analysis.

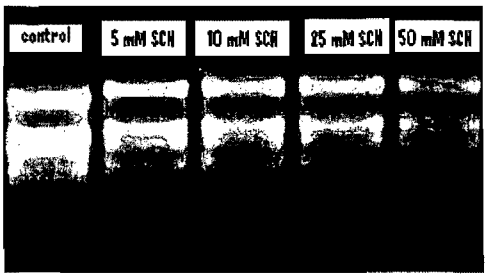


Figure 3. Checking rice RNA integrity on agarose gel after treatment with DNaseI for the removal of any contaminant DNA. Those RNA are used for differential display analysis.

**3.2 Micorarray analysis**

RNA was extracted from *A. thaliana* plants and sent to Genome Québec and McGill Innovation Center for analysis on the *Arabidopsis* ATH1-121501 Genome Chip Array. Three sets of 10 μg of total RNA — the control RNA and RNA from plants exposed to 1 mM and 2 mM $^{-}$SCN — were reverse transcribed, and hybridized with three arrays. Because of the high frequency of false positives encountered in microarray analysis, it is usually recommended to use a minimum of three replicates for each treatment. Besides, the use of many replicates allows more accurate statistical analysis for more accurate comparison in the level of expression between the control array and the tested array. The objective was to compare the gene expression patterns in control and $^{-}$SCN-exposed plants (1 and 2 mM $^{-}$SCN). The construction of between-class scatterplots was used to identify possible differential expression between control and treated samples. The between-class plots consist of the average expression of each gene in treated samples versus the expression of the gene in the control sample. Figure 4 represents a between-class plot with +> 2.5 fold change. The fold change is the ratio of the average expression in treated samples (average for 1 mM $^{-}$SCN and 2 m $^{-}$SCN-treated plants) to the control sample, or *vice versa*. The probe sets highlighted in green are those that meet the specified fold change. In the plot, the x-axis value is the control sample intensities for each probe, and the y-axis is the average of the treated sample intensities for each probe. The probes that are above

the dashed line (y=x)- about 55 probe sets- are over-expressed by a fold change of 2.5, and those that are below the dashed line-about 19 probe sets- are repressed by a fold change of 2.5.

In order to get a narrower number of differentially expressed genes, with the highest possible fold change between the control and the treated samples, the analysis was done in the same manner but with a gradual increase of fold change. It was shown that at a fold change above 350, there was no difference in expression. The only gene that showed an increase in expression in the treated samples with a 350 fold change was 266353 (Figure 5a). At a fold change of 200, genes 266353 and 253024 showed an increase in expression in the treated compared to the control samples (Figure 5b). At a lower fold of 20, a set of genes showing an increase in expression in the treated samples was chosen. These genes were identified and primers were designed to isolate and amplify these different genes from the genomic DNA of *Arabidopsis*. Table 1 lists the chosen genes. The probe ID of each gene is shown, along with its transcript ID, gene description, and fold change expression between the treated and control samples. Table 2 shows the forward and reverse primers designed for isolation and amplification of these genes. Figure 6 shows the bands obtained from the amplification of these genes.

In order to confirm that the candidate genes are induced by $^{-}$SCN, control and $^{-}$SCN-exposed RNA were subjected to RT-PCR using the primers corresponding for each gene. Genes that are more expressed after treatment should be detected earlier by PCR in samples with treated RNA. For further

confirmation, the isolated genes were used as probes for RNA blot analysis (northern blot analysis). Each probe was hybridized with a northern membrane containing a lane of control RNA and a lane of $^{-}$SCN-exposed RNA. Probes corresponding to up-regulated genes following the $^{-}$SCN treatment should hybridize more strongly with the treated RNA.

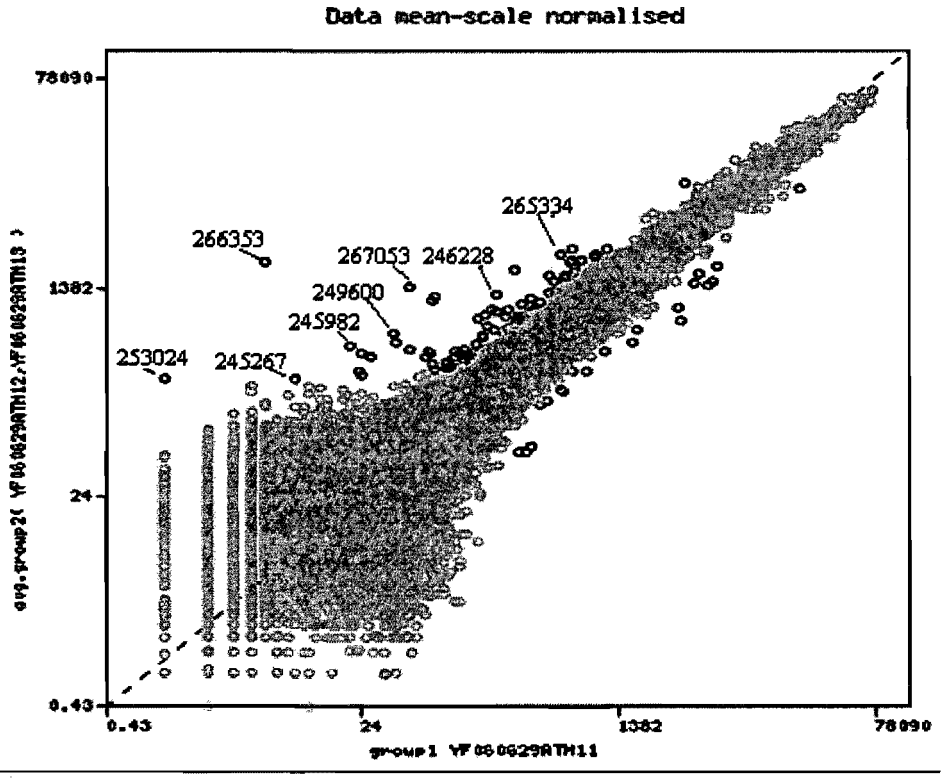


Figure 4. Microarray between-class scatterplot. Plot of the average results for the treated samples versus the result of the control sample with a fold change of +> 2.5. Probe sets highlighted in green are either over-expressed (above the dashed line) or repressed (below the dashed line) by a 2.5 average fold change in the treated samples compared to the control sample.

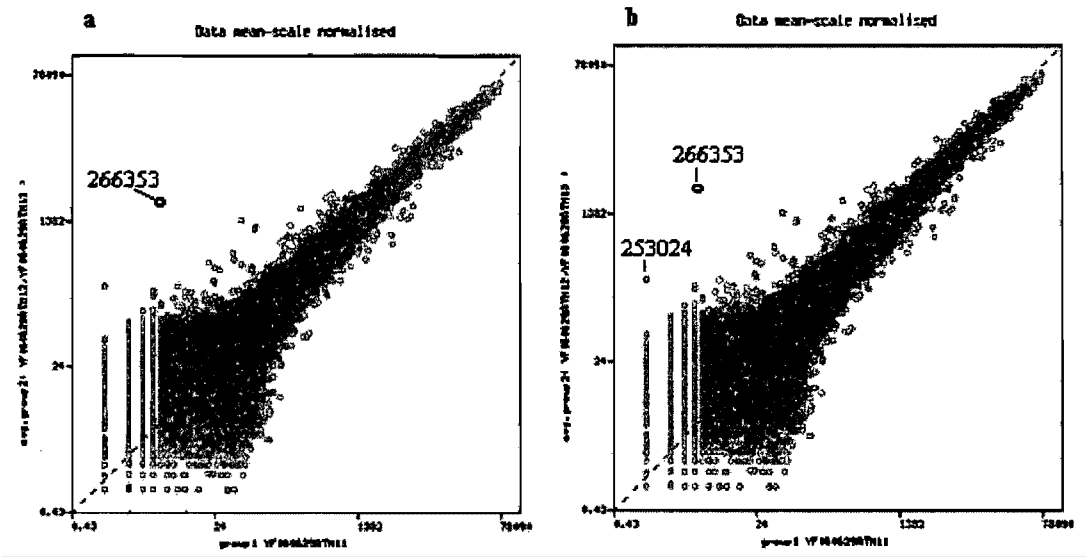


Figure. 5. Microarray between-class scatterplot. Plot of the average results for the treated samples versus the result of the control sample with a fold change of 350 (a) and 200 (b). The probe set 266353 highlighted in green is induced in the treated samples by a 350 average fold change. Probe set 253024 is induced in the treated samples by a 200 average fold change.

| Probe | Name | ID | Fold change |
|---|---|---|---|
| 266353 | major latex protein | At2g01520 | 350 |
| 253024 | hydroxyproline-rich glycoprotein | At4g38080 | 200 |
| 245982 | nodulin MTN3 protein | At5g13170 | 20 |
| 267053 | peroxidase | At2g38390 | 20 |
| 249600 | NADP-dependent oxidoreductase | At5g38000 | 10 |
| 265334 | protease inhibitor/seed storage/LTP... | At2g18370 | 10 |
| 250500 | hydroxyproline-rich glycoprotein | At5g09530 | 10 |
| 262260 | Bet v allergen | At1g70850 | 10 |
| 266393 | glycine-rich protein/late embryogenesis | At2g41260 | 5 |
| 249596 | hypothetical protein | At5g37950 | 5 |
| 246228 | peroxidase | At4g36430 | 5 |
| 245267 | major latex protein | At4g14060 | 2 |

Table I. The list of genes that showed an increase in expression upon $^{-}$SCN treatment according to the microarray analysis and that were chosen for further analysis by RT-PCR and northern blotting. For each probe, the gene name, transcript ID and fold change expression between the control and tested array is listed.

| Probe | Primer 1 | Primer 2 | Length (bp) |
|---|---|---|---|
| 266353 | CTCGAACCTTATTATGAGCTCCAA | AGTGTAGTTGGGTAGTAGTGGATT | 664 |
| 253024 | CGTGTTATGTCGTCATCATCATCG | TGACGTTGAGTTGTCTGACTTGGT | 644 |
| 245982 | TAGCCGATACTTCAACAAGTATCG | TCCGATGAGCAGGACTTTGGCAAA | 551 |
| 267053 | AAGAAGCGAGACCGATTATGTCGA | TTGCTGCTACCACAACACTCAAGA | 547 |
| 249600 | AAGATGTGACCTGAAGAACCAT | TTACAATCTCCGTCACGAGCTT | 744 |
| 265334 | TTTTGCATGCATCACGTCAGCT | CCACTGATGTAGATGTTGTGAG | 652 |
| 250500 | AGCCATCGAGACCGAAACCTTA | TTAGAGGCCATGGAAACCATTGAG | 640 |
| 262260 | TGACTTCGAGGATCAAACCACC | TACACACTAACTTCGTACGGCT | 523 |
| 266393 | AACTGGAGATCCATGGAAGAATCC | TTGTAGCAACCCGAGTTGTCAAAG | 616 |
| 249596 | TTCTCCTCGATCACGGTCTCAA | CACTTACGTTCCATGAACCGTT | 628 |
| 246228 | ACCAAATCTGGCCACCTGGTTCAA | CACCGCTTTAGTCCTTCTTAACGT | 664 |
| 245267 | TTGTATACACTGCCTCCAAGGA | GTACTTCAACCAGTTCTCAGAG | 682 |

Table II. The list of forward (primer 1) and reverse (primer 2) primers designed for each gene chosen for analysis by RT-PCR and northern blotting. The length represents the number of base pairs (bp) isolated from each gene sequence by the combination of the two primers.

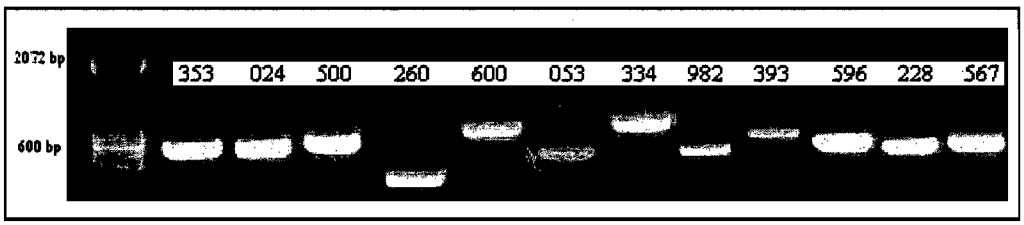


Figure 6. Agarose gel showing the sequences of candidate genes amplified by PCR based on the microarray results. The numbers represent the three last numbers of each probe ID isolated and amplified, i.e. 353 represents the probe set 266353 (gene At2g01520).

*3.2.1 Microarray analysis versus RT-PCR and northern blot analysis*

The results of microarray analysis and the comparison of expression of each probe set through scatterplot is just a preliminary indication of differential expression of genes between the treated and control samples. Genes with the highest fold change and thus with the highest probability to be differentially expressed were selected. Those genes were tested by RT-PCR and northern blot.

RT-PCR was done using RNA from control, 1 mM $^{-}$SCN and 2 mM $^{-}$SCN-exposed plants. Those RNAs were reverse transcribed and then amplified using the reverse and forward primers designed for each gene (Table 2). In some reactions, the actin2 primers were added as internal controls. Separate control reactions with the actin2 primers were also done. Equal band intensities of the actin gene in different reactions were an indication of an equal amount of RNA used in each reaction. Figure 7 shows the results obtained from the RT-PCR reactions. Unfortunately, the RT-PCR ruled out all the possible candidate genes. All the selected genes showed no change or even a decrease in the level of expression in the treated plants. The gene 266353 that had the highest fold change in the microarray chips showed the same level of expression between the control and treated RNA (Figure 7a). Another gene that had a high fold change in the microarray was 253024. The RT-PCR showed the same level of expression in control RNA and RNA of plants treated with 1 mM $^{-}$SCN, and no expression in those from plants treated with 2 mM $^{-}$SCN ($^{-}$SCN Figure 7b). The same results were observed for the other genes. Exceptions were genes in Figure 7f that were shown to be induced by $^{-}$SCN in microarray but repressed in RT-PCR. As shown

in Figure 7, the genes were expressed in control plants with a high intensity band in the control lane a, but there was no expression or a very low undetectable expression in the treated plants (no band detected in the treated lane b).

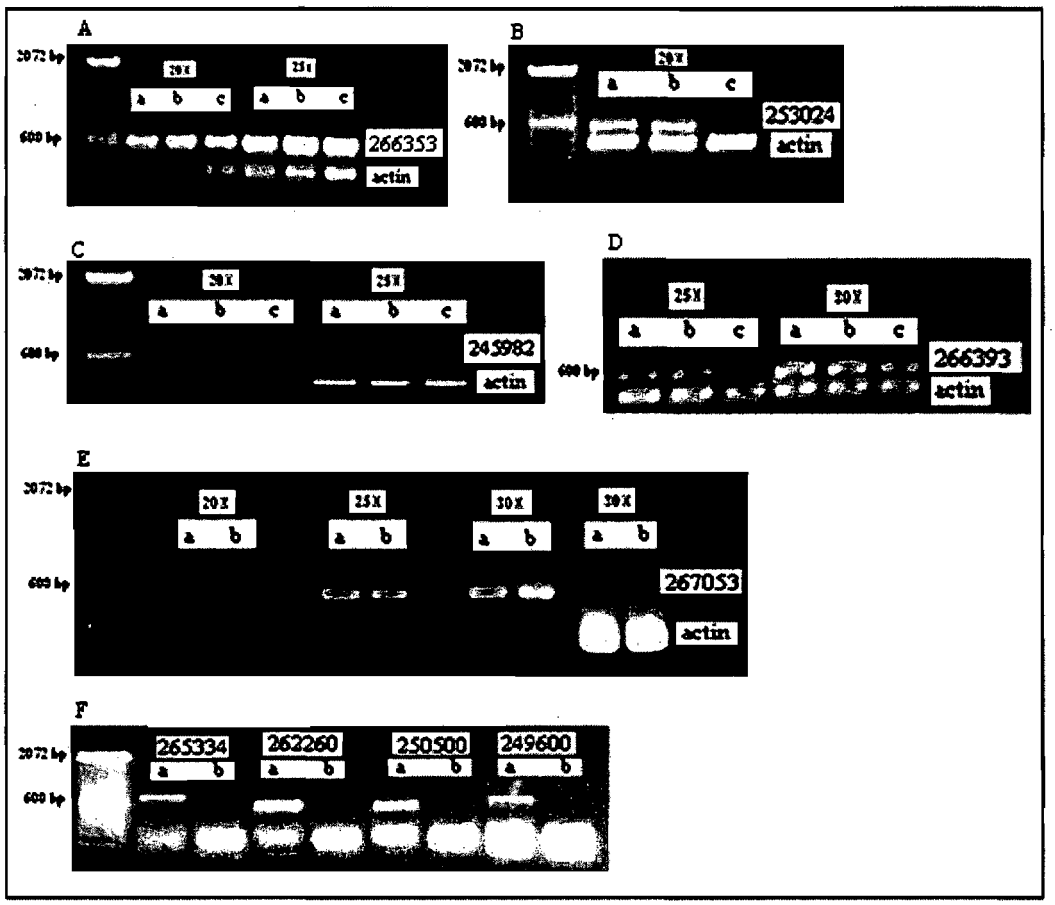
A
2072 bp
600 bp
20X
25X
a b c a b c
266353
actin
B
2072 bp
600 bp
20X
a b c
253024
actin
C
2072 bp
600 bp
20X
25X
a b c a b c
245982
actin
D
600 bp
25X
30X
a b c a b c
266393
actin
E
2072 bp
600 bp
20X
25X
30X
30X
a b a b a b a b
267053
actin
F
2072 bp
600 bp
265334
262260
250500
249600
a b a b a b a b

Figure 7. Agarose gel showing the results obtained for the RT-PCR reactions using primers designed for the amplification of candidate genes isolated based on the microarray results. Lane a: control RNA. Lane b: 1 mM $^{-}$SCN-exposed RNA. Lane c: 2 mM $^{-}$SCN-exposed RNA.

A: Probe set 266353. Bands obtained after 20 and 25 amplification cycles. The lower bands represent the control gene actin2.

B: Probe set 253024. Bands obtained after 20 amplification cycles. The lower bands represent the control gene actin2.

C: Probe set 245982. Bands obtained after amplification after 20 and 25 amplification cycles. The lower bands represent the control gene actin2.

D: Probe set 266393. Bands obtained after 25 and 30 amplification cycles. The lower bands represent the control gene actin2.

E: Probe set 27053. Bands obtained after amplification after 20, 25 and 30 amplification cycles. The last lanes represent the control gene actin2 after 30 amplification cycles.

F: Probe sets 265334, 262260, 250500, and 249600. Bands obtained after 25 amplification cycles.

In an attempt to clarify the confusion between the results from microarray and RT-PCR analysis, probes were prepared from the selected genes and hybridized with membranes containing a control RNA lane and $^{-}$SCN-treated RNA lane. Northern analysis confirmed the RT-PCR results. As shown in Figure 8, the probe sets 266353, 253024, 245982, 266393 and 267053 showed no differential expression between control and $^{-}$SCN-treated RNA. This result is consistent with the RT-PCR results. Probe sets 265334, 250500 and 249600 showed a possible decrease in expression in plants treated with 1 mM $^{-}$SCN compared to control plants. These results are also consistent with the RT-PCR but contradict the results obtained with microarray analysis.

RT-PCR and northern analysis on candidate genes selected based on microarray chips results showed that the microarray chips gave many false positives. No selected gene was shown to be induced in presence of $^{-}$SCN. Therefore, the microarray results were ignored and differential display technique, a more convenient way for the analysis of the expression profile of genes in the presence of $^{-}$SCN, was applied.

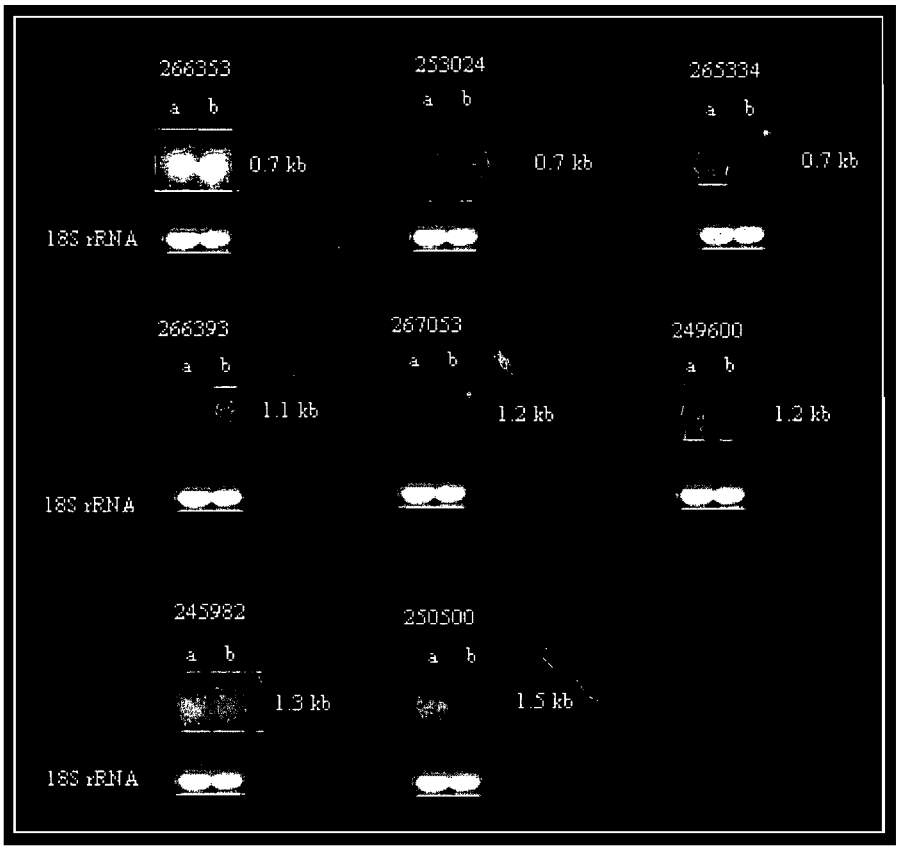


Figure 8. Northern analysis with probes prepared from candidate genes isolated based on microarray results. Lane a: control RNA. Lane b: 1 mM $^{-}$SCN-exposed RNA. Numbers above each northern membrane indicate the ID of the probe set with which each membrane was hybridized.

**3.3 Differential display analysis**

Since the microarray chips results were not convincing and showed many false positives results, an alternative technique for the analysis of difference in gene expression was chosen. Through this technique, named differential display, the expression profile of the genes of plants exposed to appropriate concentrations of $^{-}$SCN could be compared to those of control plants of rice and *A. thaliana.*

For *A. thaliana*, cDNA synthesized from RNA of control plants and plants exposed to 1 and 2 mM $^{-}$SCN were screened. For rice, cDNA from control plants and plants exposed to 5 mM, 10 mM, and 50 mM $^{-}$SCN were screened. Three anchored primers (H-$T_{11}$A, H-$T_{11}$AG, and H-$T_{11}$C) were used to divide the gene population, and 16 arbitrary primers (HAP1 to HAP16) were used to amplify sequences of arbitrary genes. The combinations of anchored and arbitrary primers were random. Figures 9 and 10 show the representative results obtained with 4 primer pairs out of 16 primer pairs tested. As shown in Figure 9, cDNA were loaded as follow for *A. thaliana*: the three consecutive lanes corresponded to cDNA from different PCR mixtures but with the same combination of anchored and arbitrary primers. The analysis of the gel revealed that many bands showed a difference in intensity between lanes "a" and "b" and between lanes "a" and "c".

For rice (Figure 10), the same loading method was used but with 4 different consecutive lanes of cDNA exposed to different $^{-}$SCN concentrations and amplified with the same primer combinations.

Eight bands in cDNA from *A. thaliana* and ten in rice showed an increase in expression upon exposure to $^{-}$SCN. These bands were extracted, re-amplified and purified for analysis. The *A. thaliana* bands extracted were assigned the names A1 to A8, and those of rice were named R1 to R10. Table 3 summarizes the characteristics of each band. The re-amplified bands were loaded on an agarose gel to determine their purity. For *A. thaliana* (Figure11a), all products showed a single band indicating that they are pure, and their sizes fit with the sizes obtained in the differential display gel. For rice (Figure 11b), only R2 and R3 showed double bands on the gel. Each of those four bands was extracted and purified from the gel separately and assigned the names, R2.a (upper band), R2.b (lower band), R3.a, and R3.b. The sizes of the rice band also fit with the sizes seen on the differential display gel indicating that these are the correct bands.

In order to remove the false positive candidates, each re-amplified band was used for further analysis by a reverse northern, although it is known that sensitivity of this technique is lower than the one obtained with a northern blot analysis. Bands that showed a possibility of an increase in expression on the slot blot membrane were further analyzed on a northern blot.

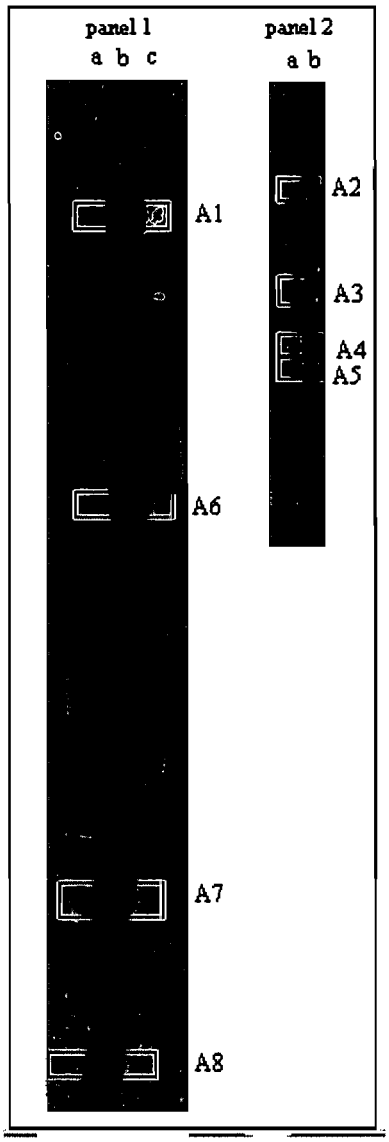


Figure 9. A partial image of a differential display gel showing the candidate fragments of *A. thaliana* cDNAs A1 to A8 that were selected and re-amplified for further analysis. Lane a: control cDNA. Lane b: 1 mM $^{-}$SCN-exposed cDNA. Lane c: 2 mM $^{-}$SCN-exposed cDNA. Panel 1: cDNA amplified from the combination of primers H-$T_{11}$A and HAP9. Panel 2: cDNA amplified from the combination of primers H-$T_{11}$C and HAP9.

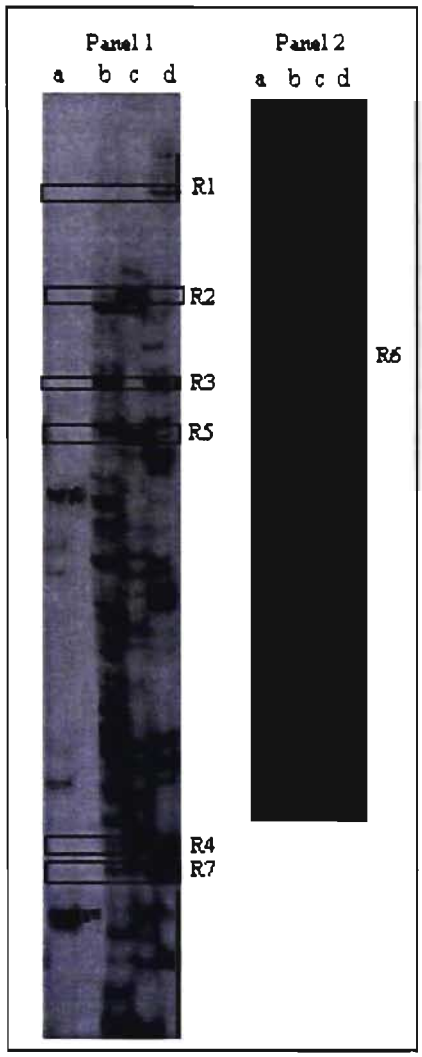


Figure 10. A partial image of a differential display gel showing the candidate fragments of rice cDNAs R1 to R7 that were selected and re-amplified for further analysis. Lane a: control cDNA. Lane b: 5 mM $^{-}$SCN-exposed cDNA. Lane c: 10 mM $^{-}$SCN-exposed cDNA. Lane d: 50 mM $^{-}$SCN-exposed cDNA. Panel 1: cDNA amplified by the combination of primers H-$T_{11}$A and HAP1. Panel 2: cDNA amplified from the combination of primers H-$T_{11}$C and HAP2.

| Band | Anchored primer | Arbitrary primer | Expression profile: Increase upon exposure to $^{-}$SCN |
|---|---|---|---|
| A1 | H-$T_{11}$A | HAP9 | 1 mM $^{-}$SCN |
| A2 | H-T11C | HAP9 | 1 mM $^{-}$SCN |
| A3 | H-T11C | HAP9 | 1 mM $^{-}$SCN |
| A4 | H-T11C | HAP9 | 1 mM $^{-}$SCN |
| A5 | H-T11C | HAP9 | 1 mM $^{-}$SCN |
| A6 | H-$T_{11}$A | HAP9 | 1 mM $^{-}$SCN |
| A7 | H-$T_{11}$A | HAP9 | 1 mM $^{-}$SCN |
| A8 | H-$T_{11}$A | HAP9 | 1 mM $^{-}$SCN |
| R1 | H-$T_{11}$A | HAP1 | 50 mM $^{-}$SCN |
| R2 | H-$T_{11}$A | HAP1 | 10 mM $^{-}$SCN |
| R3 | H-$T_{11}$A | HAP1 | 50 mM $^{-}$SCN |
| R4 | H-$T_{11}$A | HAP1 | 50 mM $^{-}$SCN |
| R5 | H-$T_{11}$A | HAP1 | 10 mM $^{-}$SCN |
| R6 | H-T11C | HAP2 | 10 mM $^{-}$SCN |
| R7 | H-$T_{11}$A | HAP4 | 50 mM $^{-}$SCN |
| R8 | H-T11C | HAP4 | 50 mM $^{-}$SCN |
| R9 | H-T11C | HAP4 | 50 mM $^{-}$SCN |
| R10 | H-T11C | HAP4 | 10 mM $^{-}$SCN |

Table III. Characteristics of candidate bands re-amplified from differential display gel. The table shows the combination of arbitrary and anchored primers used for each band and $^{-}$SCN concentration at which each band had shown an increase in expression.

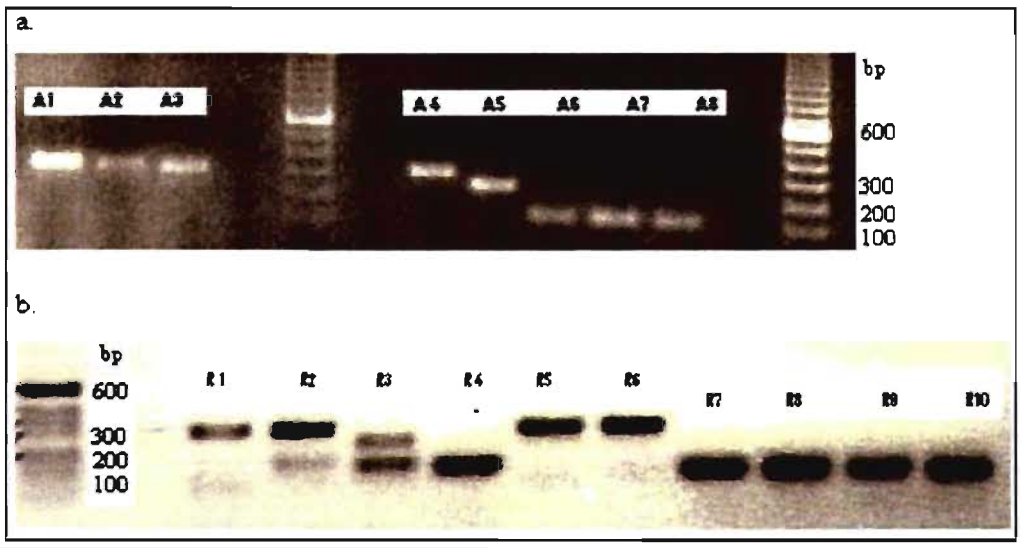


Figure 11. Re-amplification of the fragments cut out from the differential display gel. (a) Fragments A1 to A8 of *A. thaliana*. (b) Fragments R1 to R10 of rice. Fragments R2 and R3 showed two bands that were separated by extraction from the gel.

*3.3.1 Analysis of the putative bands by Slot blot*

In order to analyze the putative bands shown to be induced in the presence of $^{-}$SCN by differential display analysis, 1.5 μg of each amplified cDNA were loaded on a positively charged membrane. A replicate of each DNA was loaded and hybridized with a radioactively labeled reverse transcribed RNA probe from control plants and from plants exposed to the appropriate concentration of $^{-}$SCN. The results were not convincing since bands were also observed in the control membrane as shown in Figure 12. In the case of rice (Figure 12b), a possible increase in expression is observed for candidates R2, R4, R6, R7, and R9. Those genes were selected for further analysis by northern blotting. For *A. thaliana* (Figure 12a), a low level of hybridization was also observed in the control membrane, but results indicated that there was a possible increase in expression of all the bands in plants exposed to $^{-}$SCN, however further confirmation with northern blotting was needed.

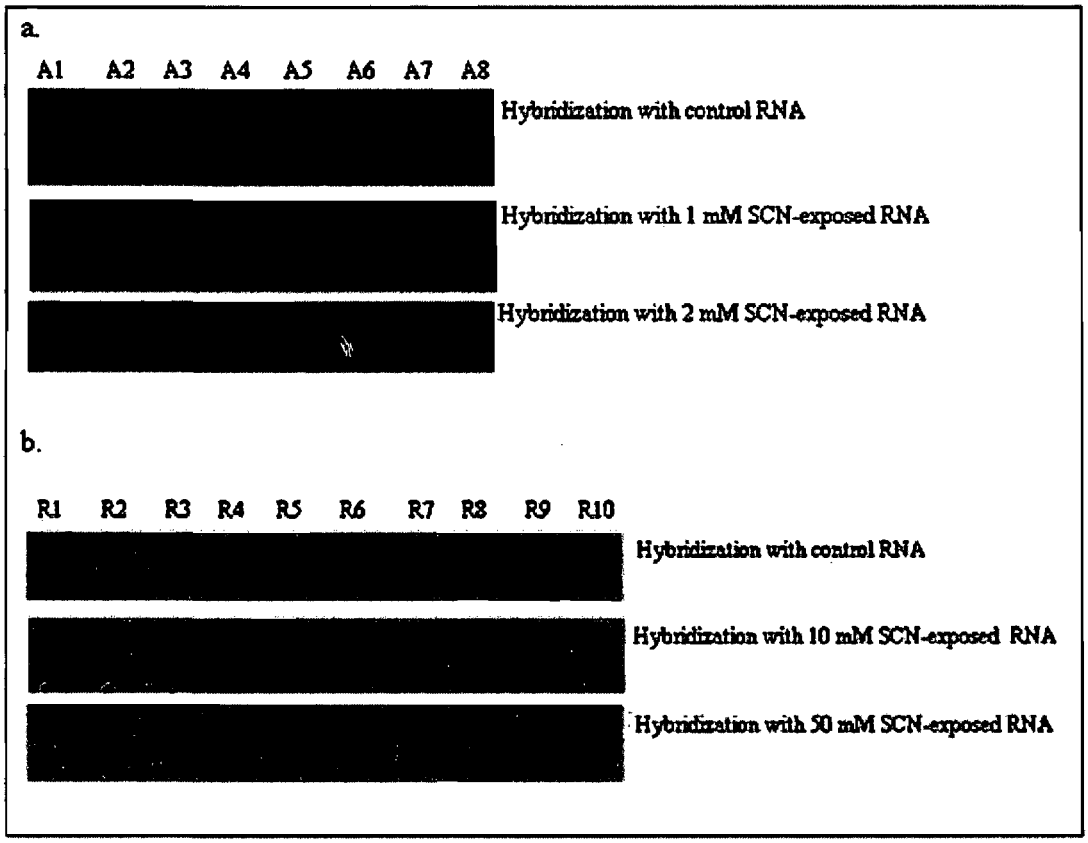


Figure 12. Reverse Northern analysis of differential display candidates. (a) Fragments A1 to A8 of *A. thaliana* hybridized with control, 1 mM, and 2 mM $^{-}$SCN-exposed RNA. (b) Fragments R1 to R10 of rice hybridized with control, 10 mM, and 50 mM $^{-}$SCN-exposed RNA.

*3.3.2 Analysis of the putative bands by northern blot*

The selected cDNA candidates were radioactively labeled and hybridized individually to their corresponding northern membranes that contained 10 µg of control and $^{-}$SCN-exposed RNA.

Since the reverse northern slot blot analysis was not sensitive enough for *A. thaliana* samples, eight membranes were prepared and hybridized with the eight different candidate probes individually. The autoradiogram showed a differential expression only in the case of the cDNA corresponding to band A6. Figure 13 shows an increase in expression of the band A6 in lane "b" (1 mM $^{-}$SCN-exposed RNA) compared to lane "a" (control RNA). For the other membranes, fragments A1 to A4 showed no difference in expression between the control and $^{-}$SCN-exposed lanes. For fragments A5, A7, and A8, the level of expression was too low and sometimes undetectable in both the control and $^{-}$SCN-exposed RNA, even after a longer hybridization time with the probe, or a longer exposure time to the film.

In the case of rice RNA, five membranes were hybridized with the five putative cDNA probes. Only a single hybridization (R4) resulted in a differential expression in the $^{-}$SCN-exposed RNA lane compared to the control RNA lane. Figure 14 shows an increase in expression of the band R4 in lane "d" (50 mM $^{-}$SCN-exposed RNA) compared to the lane "a" (control RNA). For other hybridizations (bands R2, R6, R7, and R9), no difference in the intensity of probe hybridization was observed between the control and $^{-}$SCN-exposed lane.

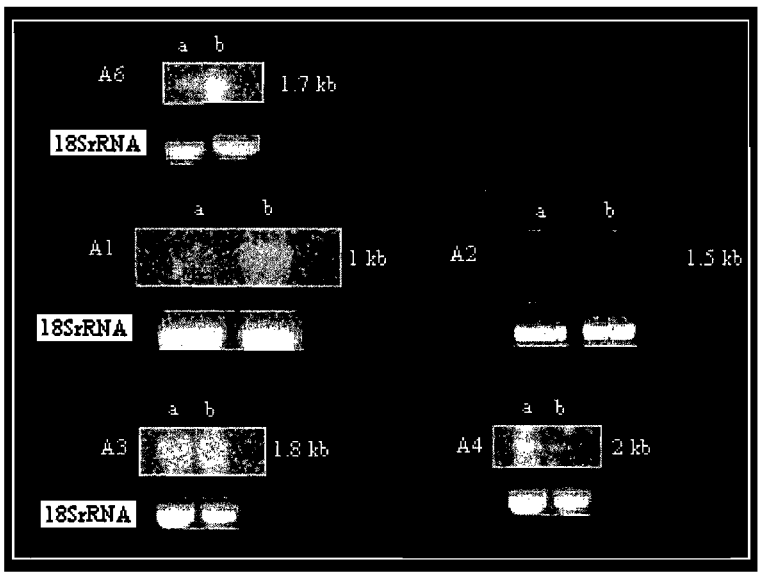


Figure 13. Northern analysis of fragments A1 to A4 and A6 of *A. thaliana*. Lane a: control RNA. Lane b: 1 mM $^{-}$SCN-exposed RNA. The fragment A6 showed an increase in expression in lane "b" compared to lane "a".

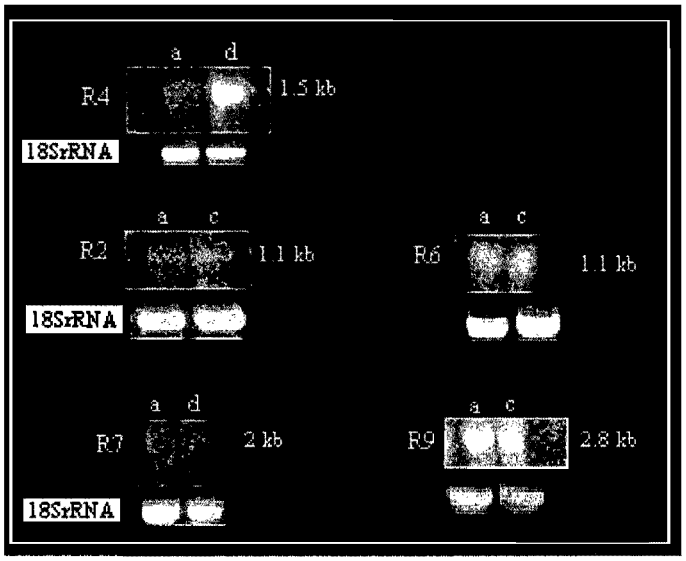


Figure 14. Northern analysis of fragments R2, R4, R6, R7 and R9 of rice. Lane a: control RNA. Lane b: 5 mM $^{-}$SCN-exposed RNA. Lane c: 10 mM $^{-}$SCN-exposed RNA. Lane d: 50 mM $^{-}$SCN-exposed RNA. Only fragment R4 showed an increase in expression in lane "d" compared to lane "a".

**3.4 Sequencing of differentially expressed genes**

The differential display analysis for *A. thaliana* and rice cDNA showed multiple candidate bands that might correspond to genes differentially expressed following $^{-}$SCN treatment. Northern blot analysis confirmed that two of these candidate bands showed an increase in expression following treatment: band A6 of *A. thaliana* and R4 of rice. These two bands were purified and re-amplified for sequencing.

The sequencing of bands A6 and R4 and the BLAST analysis of the resulting sequences in GenBank showed 100% homology between A6 and a BSD domain-containing protein (At1g03350) on chromosome 1 of *A. thaliana*, and between R4 and the Os09g0442300 gene on chromosome 9 of rice. The alignments of the forward and reverse sequences of each fragment with the corresponding genes and chromosome sequences are shown in Figure 15 for *A. thaliana* and Figure 17 for rice. The full sequence of At1g03350 gene is shown in Figure 16 and that of Os09g0442300 gene is presented in Figure 18.

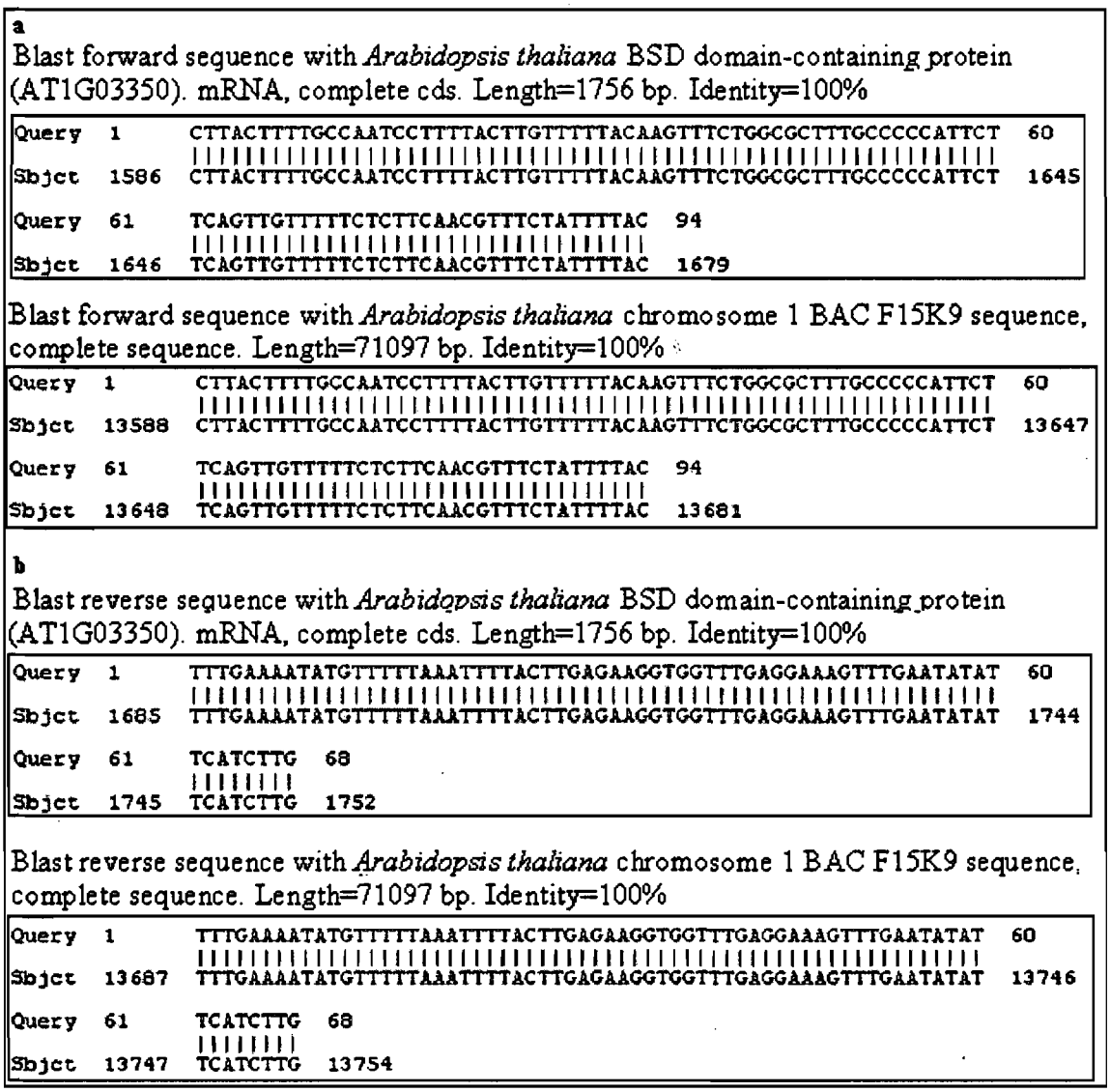


Figure 15. BLAST of forward and reverse sequences of fragment A6 with the *A. thaliana* genome. Searching the nucleotide database showed 100% sequence identity with At1g03350 gene on chromosome 1 of *A. thaliana*. (a) BLAST of the forward sequence with At1g03350 gene and *A. thaliana* genomic DNA, chromosome 1 (100% homology). (b) The BLAST of the reverse sequence with At1g03350 gene and *A. thaliana* genomic DNA, chromosome 1 (100% homology). Query: the sequence obtained from the differential display analysis. Subject: the known *A. thaliana* sequence.

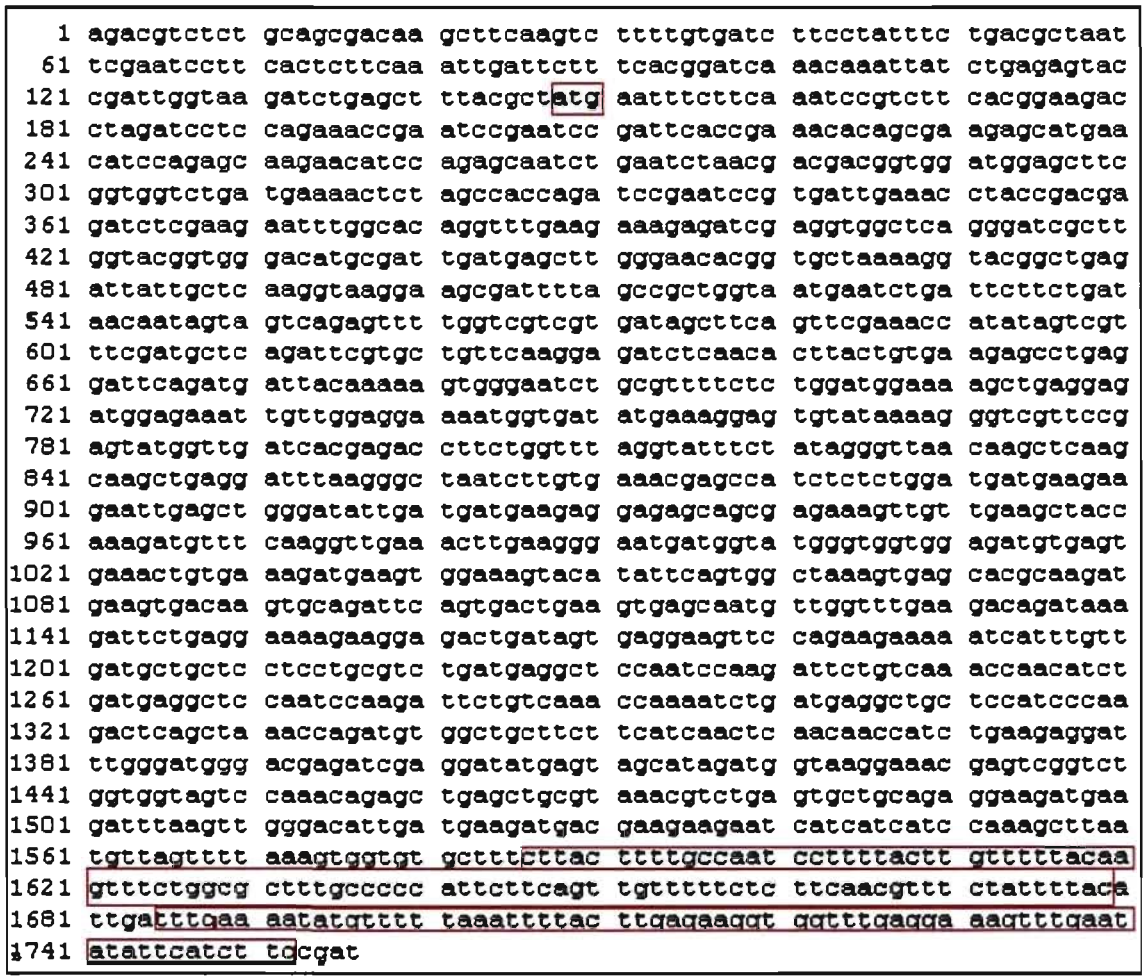


Figure 16. *A. thaliana* BSD domain-containing protein (At1g03350) mRNA complete sequence. The start codon ATG is highlighted in red. The sequence from the band obtained from the differential display analysis is highlighted in red (162 bp).

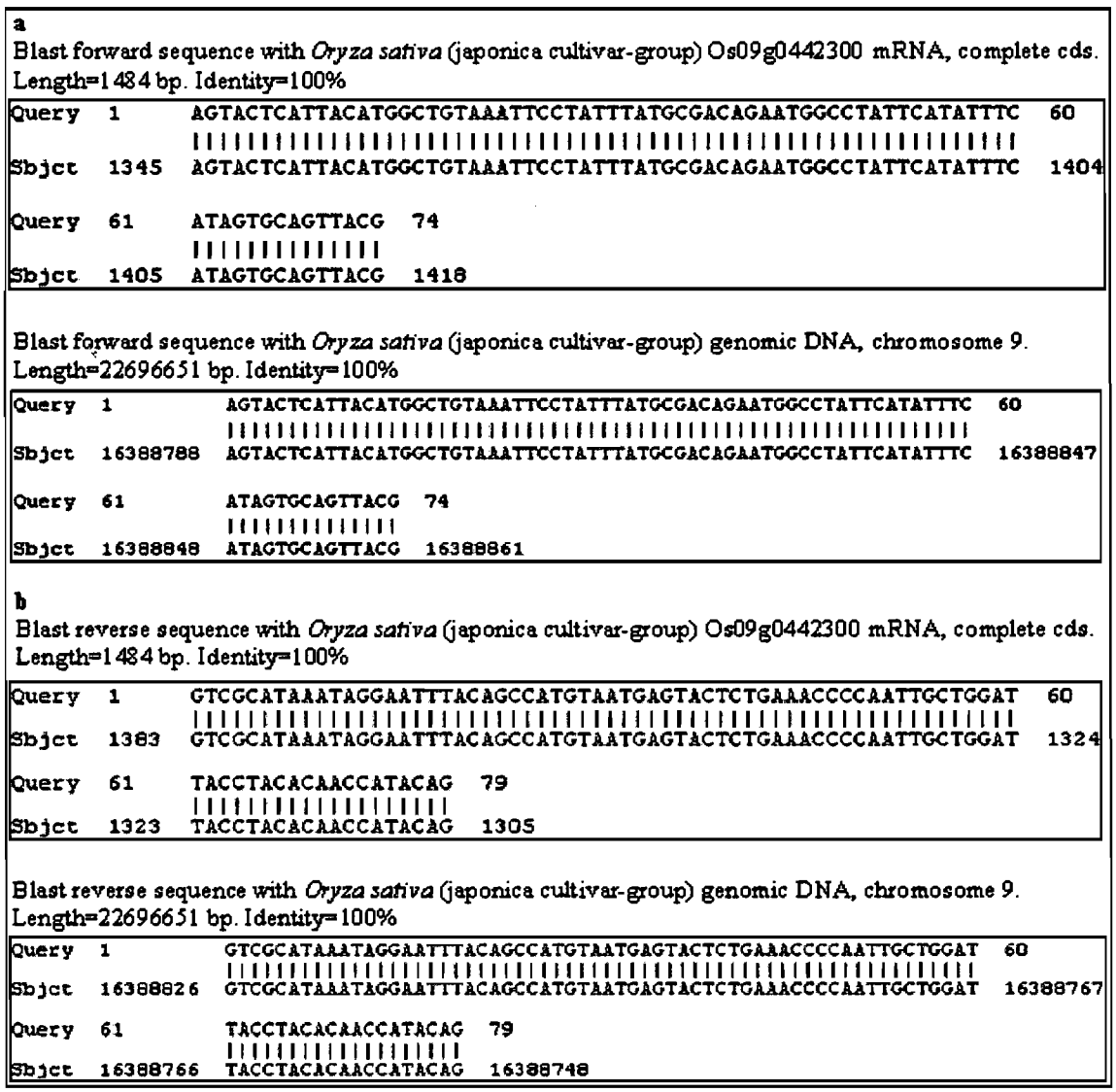


Figure 17. BLAST of forward and reverse sequence of fragment R4 with the rice genome. Searching the nucleotide database showed 100% sequence identity with Os09g0442300 gene on chromosome 9 of rice. (a) BLAST of the forward sequence with Os09g0442300 gene and rice genomic DNA, chromosome 9 (100% homology). (b) BLAST of the reverse sequence with Os09g0442300 gene and rice genomic DNA, chromosome 9 (100% homology). Query: the sequence obtained from the differential display analysis. Subject: the known *O. sativa* sequence.

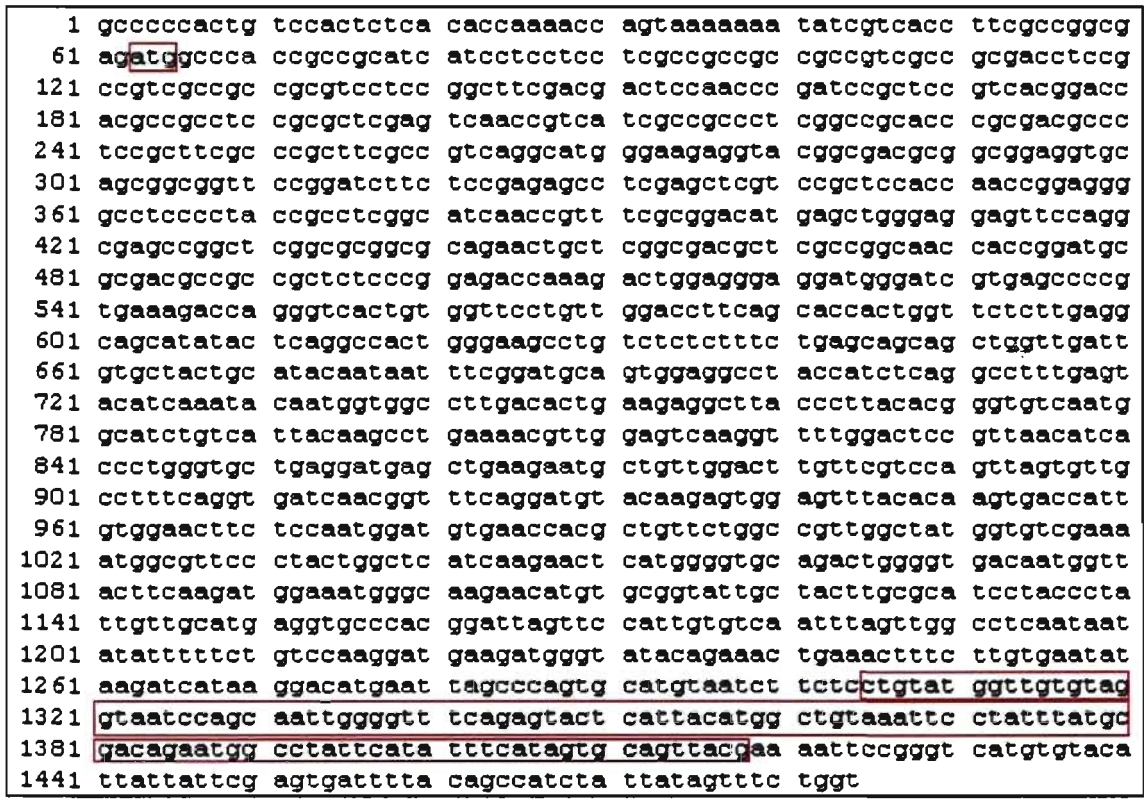


Figure 18. *O. sativa* (Japonica cultivar-group) Os09g0442300 full sequence. The start codon ATG is highlighted in red. The band obtained from differential display analysis is highlighted in red (153 bp).

**3.5 $^{-}$SCN-inducible promoter isolation**

The availability of the full genome sequence of both plants allowed access to the upstream sequence of the $^{-}$SCN-induced genes. My goal was to isolate the upstream sequence that contains the promoter responsive to $^{-}$SCN. For the Os09g0442300 gene of rice, several trials for the access to the upstream sequence of the gene by PCR led to smears or multiple bands on the agarose gel. Changes in different parameters for the PCR reactions and in the sequence itself failed to solve the problem. The use of different PCR programs (change in the primer annealing temperature, extension time), different concentrations of DNA, enzymes, magnesium concentrations, or primer sequences and combinations failed to overcome the smear or multiple bands observed on the gel. For the primer sequences, primers RUF and RUR (section 2.11) were used as the major primers. Other primer sequences and combinations were also used for the optimization of the reaction conditions. These sequences are listed in the Table IV. Figure 19 shows the effect of the different parameter changes on the PCR product obtained. Beside these changes, it was later found that the genomic DNA used is extracted from the rice of the type Indica, and the primers were designed for the sequence of the rice of the type Japonica. DNA was extracted from the Japonica rice in the hope of solving the problem. The PCR led to a more defined band at the expected size (2067 bp), but other bands were always present at different sizes. The band at 2067 bp was extracted from the gel but attempt for reamplification of the band failed again.

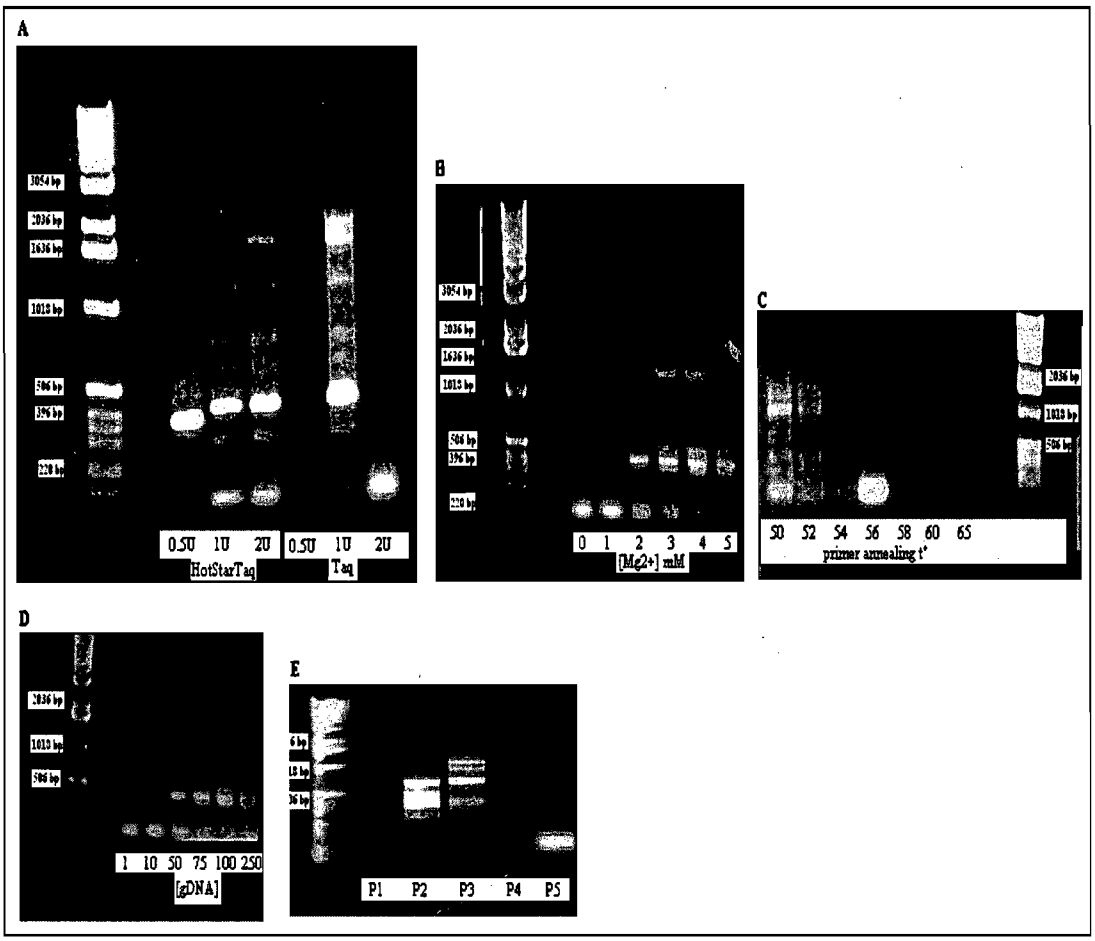


Figure 19. Agarose gels showing the bands obtained from the different PCR trials done to isolate the sequence upstream of Os09g0442300 gene of rice.

A-D: primers RUF and RUR used, and a fragment of 2067 bp was expected.

A: use of different enzyme concentrations (0.5U, 1U, and 2U) and two different enzymes (Taq polymerase and HotStarTaq polymerase)

B: Addition of different concentrations of the ion $Mg^{2+}$ (0 to 5 mM)

C: Use of different temperatures for the annealing of the primers (50°C to 65°C).

D: Use of different genomic DNA concentrations (1 ng to 250 ng)

E: Use of other primer combinations generating different fragment sizes listed in table 4.

| Combination | Primer 1 | Primer 2 | Length (bp) |
|---|---|---|---|
| P1 | GAGAGGATCCGCTAGCAGGTAGTATTGCAGTTT | GAGACCATGGTTGGTTTTGGTGTGAGAGTGG | 1515 |
| P2 | GAGAGGATCCCCGGGAGAATAAAATGGACA | GAGACCATGGGCTGGCCACTGGTATGACTT | 3335 |
| P3 | GAGAGGATCCAAAACTACGGCGGCTAAACA | GAGACCATGGGCTGGCCACTGGTATGACTT | 2588 |
| P4 | GAGAGGATCCTCTTGGACACTTGCAGATCG | GAGACCATGGGCTGGCCACTGGTATGACTT | 1842 |
| P5 | GAGAGGATCCTGCAGCCGATCCAATGAACC | GAGACCATGGGGGCAGGAAGCTTGGATGGA | 2192 |

Table IV. Primer combinations used for the amplification of the upstream sequence of Os09g0442300 gene of rice. The table shows the forward (primer 1) and reverse (primer 2) used and the length of the amplified sequence.

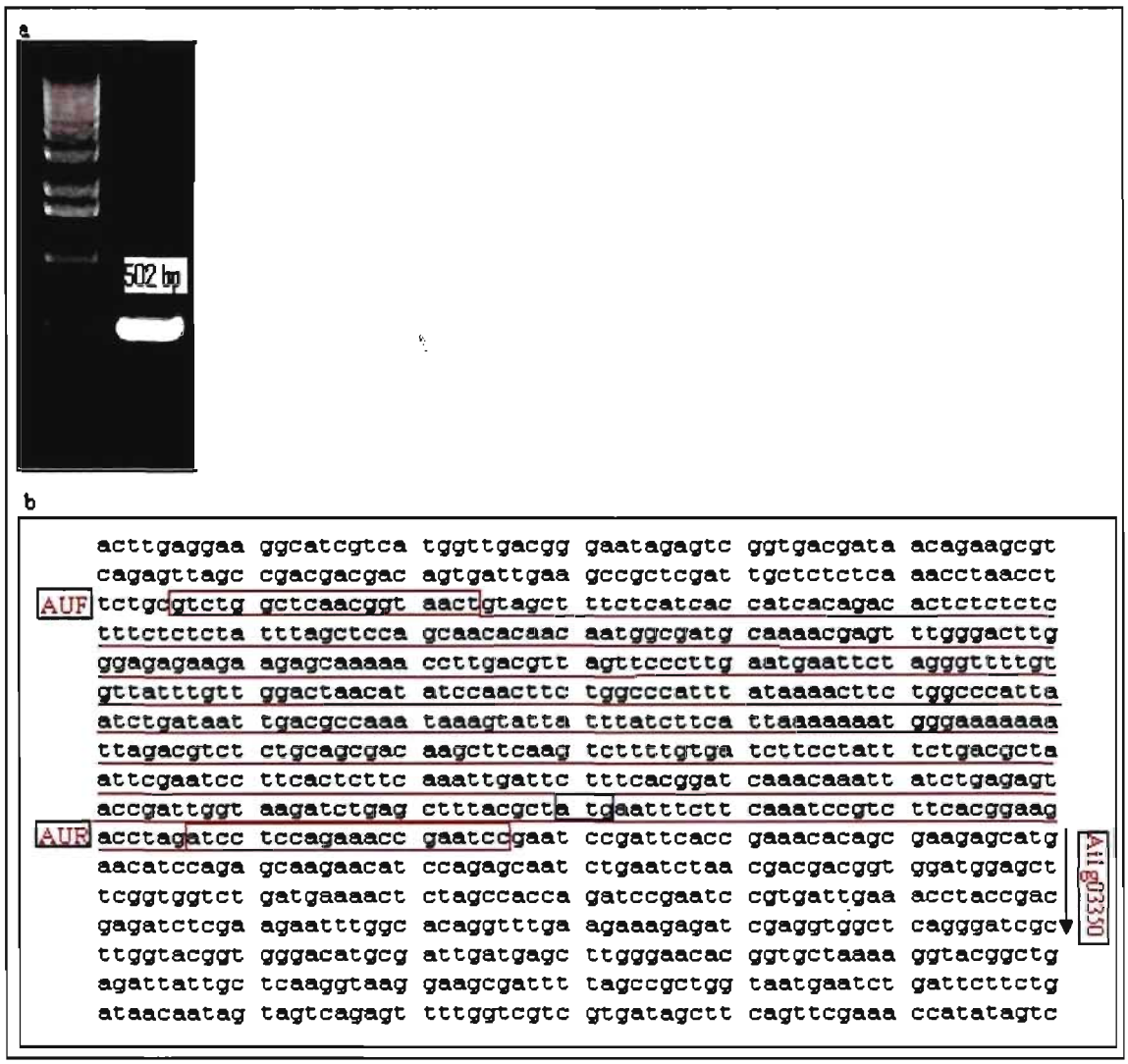


Figure 20. PCR amplification of the BSD domain-containing protein (At1g03350) upstream sequence with the primers AUF and AUR. (a) The PCR product electrophoresed on agarose gel showing a pure band at 502 bp. (b) The nucleotide sequence of the amplified upstream sequence is underlined in red with the two primer sequences AUF and AUR highlighted in red. The start codon ATG of At1g03350 is highlighted in blue.

In the case of *A. thaliana*, a 502 bp sequence upstream of the gene encoding BSD domain-containing protein (At1g03350) was successfully isolated and amplified by PCR using the primers AUF and AUR (Figure 20a). This sequence was expected to contain the $^{-}$SCN-induced promoter. Usually, the promoter region in dicots is found 1000 or 2000 bp upstream of the transcription start site. In this case, another gene, At1g03360 is found just about 500 bp upstream of At1g03350, indicating that the sequence containing the regulatory element of the $^{-}$SCN-induced gene is found in the 500 bp region. Figure 20b shows the amplified sequence highlighted in red with the two primers used for the amplification.

## 3.6 Bacterial Transformation

### *3.6.1 E. coli transformation*

In order to test the *A. thaliana* upstream sequence isolated for the presence of the promoter induced by $^{-}$SCN, the sequence was first introduced into a promoterless binary vector, pCambia 1291Z. It was inserted in the multiple cloning site (MCS), upstream of the reporter gene, *gusA*. pCambia 1291Z vector and the position of *gusA* gene is shown in Figure 21. The construct was then transformed in *E. coli* for multiplication of the plasmid. Figure 22 shows the positive clones containing the vector with the inserted sequence.

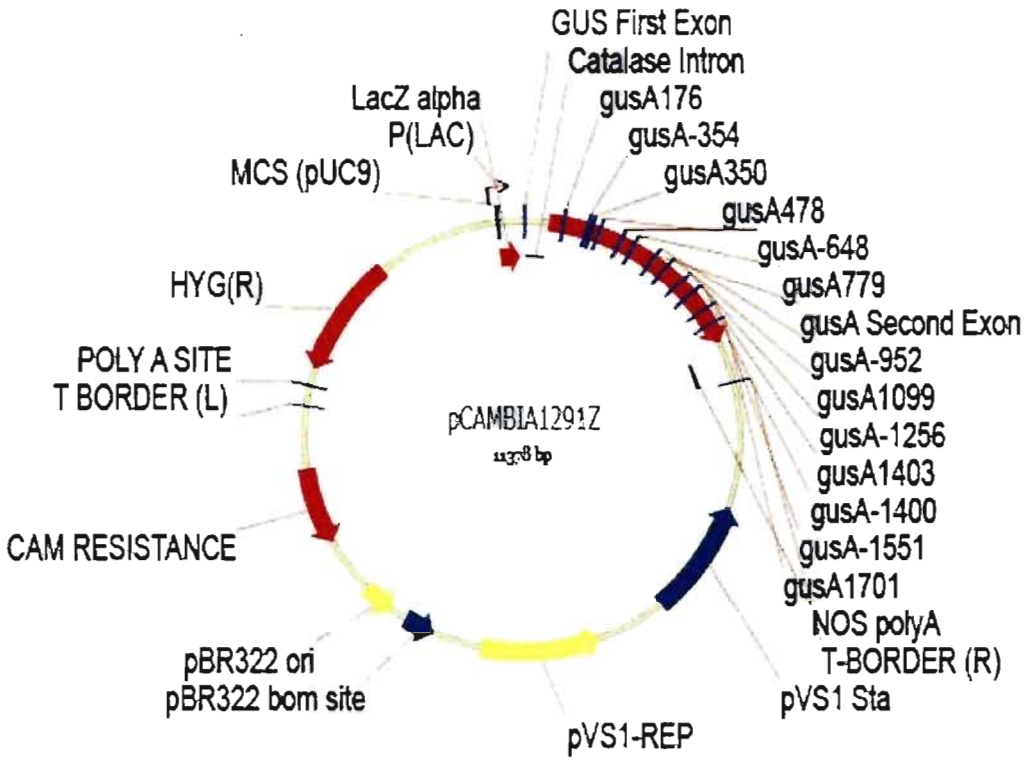


Figure 21. Genetic map of the binary vector pCambia1291Z containing the reporter gene *gusA*.

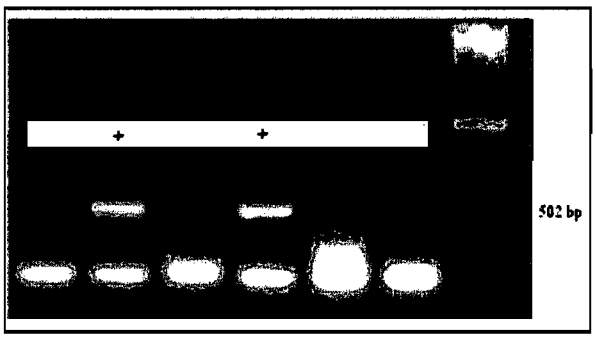


Figure 22. Agarose gel showing the PCR products obtained from the amplification of the plasmid by the primers AUF and AUR. The plus sign indicates positive clones of the construct pCambia1291Z-*A. thaliana* upstream sequence.

*3.6.2 Agrobacterium transformation*

The plasmid that was selected and stored had the upstream sequence of the gene At1g03350 inserted upstream of *gusA* gene. This sequence was expected to contain the promoter region that could be sufficient to control the expression of At1g03350. This promoter is expected to be induced by $^{-}$SCN since the gene showed an increase of expression upon treatment with 1 mM $^{-}$SCN.

In order to confirm that this promoter was induced by the ion, the plasmid was stably transformed into two plants, tobacco roots for quick results and into *A. thaliana*, and tested for promoter activity in the presence and absence of $^{-}$SCN by measuring GUS activity. To achieve this, the plasmid was transformed into *A. rhizogenes* and *A. tumefaciens*. Bacteria that grew on the selective media were selected and the plasmid was extracted. The extracted plasmid was amplified with the two primers AUF and AUR to test for the presence of the insert. Figure 23 shows the positive clones obtained for both *Agrobacteria* strains. Those clones were stored and maintained to be later transformed into plants.

The transformations were done using protocols described in sections 2.15, 2.16, and 2.17 but were not finalized because of lack of time. Root transformation resulted in possible transgenic roots that were grown in $^{-}$SCN-containing media and screened for GUS expression. A large number of transgenic roots were screened but no blue color was detected in any root parts. *A. thaliana* protoplast transformation failed because the protocol needed more optimization; *A. thaliana* plants growth conditions should be optimized to get healthier leaves and a higher yield of protoplasts, and the conditions of the maintenance of the protoplast

survival during transfection should also be optimized. No time was available for the optimization, and the experiment was ignored. For *A. thaliana* transformation through floral dip method, the protocol was interrupted at the first generation of seeds because of lack of time.

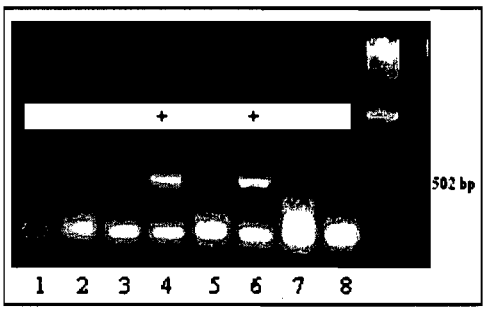


Figure 23. Agarose gel showing the plasmid extracted from *A. rhizogenes* (lanes 1-4) and *A. tumefaciens* (lanes 5-8) and amplified with the primers AUF and AUR to check for the presence of the insert. The plus sign indicates positive clones of the plasmid.

# 4. Discussion

Selectable marker genes are used in genetic transformation for the identification of positively transformed cells. The *TMT* gene is a newly developed selectable marker. The gene codes for a thiol methyltransferase (TMT) enzyme that methylates and detoxifies different thiol compounds, including $^{-}$SCN (Attieh et al., 2000). *TMT*, used together with its product's substrate $^{-}$SCN as the selective agent, has been shown to be a selectable marker that is at least as efficient as the currently prevalent marker *nptII* (Koonjul et al, unpublished). *TMT* is naturally present in plant species (Saini et al., 1995). Therefore, the use of *TMT* as a selectable marker overpasses the concerns about the introduction of foreign genes into species while using antibiotic-resitance genes as markers (Stewart et al., 2000; Miki and McHugh, 2004). However, there are still concerns about any other side effect of the selectable marker on the transformed cells even if it is found naturally in some plant species. Many strategies have been deployed to overcome these concerns and develop marker-free plants. One of these strategies is the use of chemically-induced site-specific recombinases to excise the marker genes after the selection process has been achieved (Hare and Chua, 2002).

In this study, the aim was to find a possible alternative solution to overcome any side effect of selectable markers such as the use of an inducible marker, instead of excising the marker with an inducible recombinase. Currently, no selectable marker is available that can be turned on only for selection and then turned off after the transformation. If the marker *TMT* could be regulated by its substrate $^{-}$SCN, then during the selection, the gene would be expressed in the

presence of the ion and thus would work as a selectable marker. After the selection process is completed, gene expression would be turned off as $^{-}$SCN would be absent.

The aim of this work was to identify and isolate any gene that is induced by $^{-}$SCN. The promoter of this gene could then be isolated and used to regulate *TMT* gene expression, and thus constructing an inducible selectable marker for the selection and identification of transgenic cells.

## 4.1 Identification of $^{-}$SCN-induced genes

Microarray and differential display analyses were used for the identification of possible $^{-}$SCN-induced genes in *A. thaliana* and rice. Microarray analysis resulted in a large number of false positive genes since the RT-PCR and northern blot analyses on those genes were not consistent with the microarray results. The microarray candidate genes showed either no change in expression in $^{-}$SCN-treated plants compared to control plants or, for some of these genes, a reduction in expression was even detected when plants were treated with $^{-}$SCN. The high frequency of false positives from microarray chips was not surprising because only one replicate of control and treated samples were used. A single replicate was used because the chips were expensive and the use of two $^{-}$SCN concentrations were considered as two replicates for the treated sample. However, usually it is recommended to use multiple replicates. For each sample, a within-class scatterplot is constructed plotting replicates of the same sample. Ideally, the plot should result in probe sets only along the y=x line because each probe is

expected to have the same intensity because it is exposed to the same conditions. However, the within-class plot often results in differences in probe intensities due to random variation that are not related to treatment effects. These probe sets are considered as outliers and excluded from the analysis because the variation in their expression is most probably erroneous. In the present work, the use of a single replicate did not allow the construction of within-class scatterplot and possible outliers were not excluded. Only the between-class scatterplot results were taken into consideration which led to a high frequency of false positives.

The analysis of candidate genes obtained from microarray chips was not effective. Because of the high costs of the microarray chips and the high number of replicates needed for significant statistical analysis, the differential display analysis was used as an alternative to compare expression of genes in control versus $^{-}$SCN-treated plants in *A. thaliana* and rice. The display gel resulted in multiple candidate fragments in both species. Reverse northern analysis on the differential display fragments was not sensitive enough. The band intensities on the membrane after hybridization were confusing and sometimes inconsistent with the differential display results. Usually reverse northern is known to be less accurate than northern analysis because the RNA of the organism hybridizes with a small cDNA fragment. The probability of accurate hybridization is less than that of northern blot, in which the small cDNA fragment is hybridizing with the RNA of the organism. Therefore, northern blot analysis was used to confirm the differential display results. Candidate fragments were hybridized with RNA from control and $^{-}$SCN-treated plants. Two genes showed an increase in expression in

plants treated with $^{-}$SCN. In *A. thaliana*, a BSD domain-containing protein (At1g03350) showed an increase in expression when seeds and growing plants were treated with 1 mM $^{-}$SCN. The autoradiogram showed a band with high intensity at a size of about 1.7 kb that is consistent with the size of the gene (1756 bp). In rice, the Os09g0442300 gene showed an increase in expression when plants were treated with 50 mM $^{-}$SCN for one day. The band obtained on the autoradiogram had a size of about 1.5 kb that is close to the gene size (1468 bp).

The BSD domain-containing protein (At1g03350) of *A. thaliana* is located on chromosome 1. Its molecular function is still unknown. The BSD domain is found in transcription factors, synapse-associated proteins, and different hypothetical proteins (Doerks et al., 2002). The domain is found in several species from primary protozoan to human, indicating that it is conserved among species, an indication of a probable important role. This role is still not known but findings suggest that it might be related to chromatin-associated processes such as DNA binding, since it is found in transcription factors. Other roles could also be attributed to this domain: it is sometimes present next to U box domains involved in ubiquitination, and BTB domains involved in protein-protein interactions. Further structural studies should help study the involvement of the BSD domain in these different processes. However, there is no evidence for any functional relation to $^{-}$SCN or TMT substrates on the expression of this gene.

In rice, the Os09g0442300 gene is located on chromosome 9. This gene encodes a cysteine proteinase called oryzain gamma (Watanabe et al., 1991). In

rice, there are three oryzains: oryzains α, β, and γ. They are similar to many cysteine proteinases, such as papain and cathepsin H. Oryzain γ sequence is 85% similar to aleurain and 60% similar to cathepsin H sequences. The expression of the three oryzain genes was shown to be induced only during germination by the hormone gibberellin (Watanabe et al., 1991). Our result showed that the oryzain γ expression can also be induced after germination by the ion $^{-}$SCN.

## 4.2 Identification of $^{-}$SCN-induced promoters

In general, the expression of each gene is regulated by a promoter region found upstream of this gene. When a gene is induced by an internal or external stimulus, the stimulus triggers a cascade of reactions that activates or deactivates the promoter region leading to an increase or decrease in the expression of the gene. In this study, it was found that $^{-}$SCN is a stimulus that activates the promoter regions of At1g03350 and Os09g0442300 genes and thus leading to an increase in the expression of these genes.

For the *A. thaliana* gene, an upstream sequence of 502 bp was amplified by PCR. This sequence is expected to contain the $^{-}$SCN-induced promoter region. Although promoters can be quite long and sometimes exceed a few thousand base pairs, the presence of a close neighboring gene (At1g03360) upstream of our $^{-}$SCN-induced gene (At1g03350) narrowed our target to this intergenic sequence. Thus the chosen sequence as the putative promoter comprises the region between the At1g03350 gene and the At1g03360 gene.

For rice, many trials to amplify the upstream sequence expected to contain the $^{-}$SCN-induced promoter region failed. The PCR product obtained was not pure and optimization of PCR conditions did not overcome the multiple bands obtained on the agarose gel. The use of DNA of plants of the same cultivar (Japonica Nipponbare) used in Genbank gave more defined bands but did not entirely solve the problem of multiple bands. The expected bands were excised and purified from the gel, but re-amplification or cloning of these bands failed. The rice result was then ignored for the moment, and further work was limited to *A. thaliana* induced promoter.

## 4.3 $^{-}$SCN–induced promoter analysis through GUS assay

In order to ensure that the amplified *A. thaliana* sequence contains the promoter region induced by $^{-}$SCN, the sequence was inserted into pCambia 1291Z, a binary promoterless vector that contains a reporter gene, *gusA*. The sequence was inserted upstream of *gusA* gene to regulate its expression. If in the presence of the selective agent $^{-}$SCN the promoter is activated, it will induce the expression of *gusA* that encodes the enzyme GUS. GUS cleaves X-gluc resulting in a blue color. In the absence of $^{-}$SCN, the promoter is inactive, turning off *gusA* expression, and an enzymatic assay with the X-gluc substrate will not result in a blue color.

To assay the activity of the promoter, the plasmid (pCambia 1291Z with the inserted *A. thaliana* upstream sequence) was transformed into *A. rhizogenes* and *A. tumefaciens.*

*4.3.1Possible reasons for the failure of plant transformation*

Tobacco root transformation with *A. rhizogenes* resulted in fast-growing roots in the selective medium. Those roots were screened for the expression of *gusA* gene through the addition of X-gluc. Unfortunately, SCN-exposed transgenic roots did not give a blue color in the presence of X-gluc, even after the optimization of the GUS assay conditions, i.e, changes in incubation time and temperature, in X-gluc concentrations, and in buffer composition and concentration. Because of time constrains, only the construct containing the fusion of the promoter with GUS was transformed. Negative (pCambia 1291Z vector) and positive (fusion of 35S promoter with GUS) controls were not included. Because of the absence of the controls, the possible reasons for the experimental failure could not be predicted.

One probable reason for the absence of coloration is the improper upstream sequence isolated and inserted upstream of *gusA* gene. It is possible that the promoter region is not found upstream of the gene. It might be found at the 3' downstream region. Several downstream regulatory elements have been already reported, especially in Drosophila and some human genes (Burke and Kadonaga, 1997; Kutach and Kadonaga, 2000). These elements are called DPE (downstream promoter elements) and are found downstream of the transcription start sites of genes that lack the upstream TATA box region.

Another probable reason for this experimental failure was the positions of the primers AUF and AUR designed on chromosome 1 of *A. thaliana*. Primer AUR

was designed after the start codon ATG of At1g03350 (Figure 22b). The promoter will most probably start the expression from this start codon rather than the start codon of *gusA* gene. Although translation might proceeds to *gusA* gene, there is a probability of frameshift mutation. For this reason, new primers were designed to be located in the non-coding region of At1g03350. Primer APF: 5'-GCgtcgacCACAGACACTCTCTCTCTTTCTC and primer APR: 5'-GCggatccGTAAAGCTCAGATCTTACCAATCG. APF contains the underlined restriction site for *Sal*I restriction enzyme, and APR starts with the underlined restriction site for *Bam*HI. Figure 24 shows the sequence of *A. thaliana* chromosome 1 with the two new primers APF and APR highlighted in red. The blue highlights represent the start codon ATG of At1g03350 found after APR and the start codon of At1g03360 found upstream of APF. This indicated that the sequence amplified by APF and APR will contain only the non-coding region that is expected to contain the promoter of At1g03350.

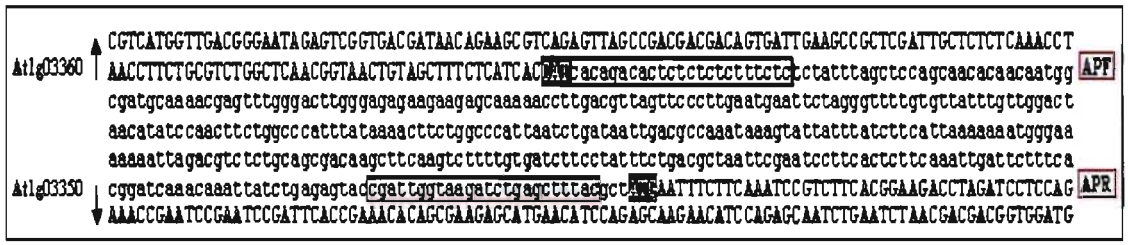


Figure 24. Sequence of *A. thaliana* chromosome 1 showing the primers APF and APR designed to amplify the upstream sequence of the ¯SCN-induced gene At1g03350. The primers are highlighted in red. The start codon of At1g03350 and the upstream gene At1g3360 are highlighted in blue.

In the future, further work on this study might involve the amplification of the sequence with the new primers. The work could be repeated by inserting the new sequence upstream of *gusA* gene in pCambia 1291Z using the restriction enzymes *Sal*I and *Bam*H1. The plasmid could be again transformed into tobacco roots and *A. thaliana* plants. Transgenic roots that have inserted the correct plasmid could be selected and subjected to GUS assay. Transgenic clones could be either exposed to $^{-}$SCN or would grow in a normal medium. In the presence of X-gluc, roots that are exposed to $^{-}$SCN are expected to show a blue color under the dissecting microscope, in contrast to roots growing in normal medium that are expected to stay colorless. Besides, *A. thaliana* transgenic plants could also be subjected to the same GUS assay and expected to give similar results. If the expected results are obtained, the proof that this promoter is induced by $^{-}$SCN would allow its use to induce the expression of *TMT* gene.

## 4.4 Development of inducible selectable marker *TMT*

The promoter that was able to induce *gusA* expression only in the presence of $^{-}$SCN could be used to regulate the expression of the selectable marker gene, *TMT*. Further stable transformations are required to test the expression of *TMT* under the regulation of the $^{-}$SCN-inducible promoter, leading to the development of an inducible selectable marker.

A future approach for the aim to develop the inducible selectable marker would be the construction of a chimeric gene. A convenient way to construct the chimeric gene is to use the pCambia vector that has the promoter inserted

upstream of *gusA* gene. *gusA* gene could be excised by appropriate restriction enzymes and *TMT1* gene inserted in its place. The promoter would be thus able to regulate *TMT1* expression. The plasmid could be transformed into *E. coli* for the preparation of a stock. The plasmid that has the correct insert could be transformed again into *A. rhizogenes* for transformation of plants yielding fast-growing transgenic roots. Roots that grow normally on the selective medium could be selected and screened for the presence of the chimeric gene. The transgenic roots could then grow in the presence or absence of $^-$SCN. TMT activity could be assayed. A convenient way to assay the TMT activity is to measure the level of $CH_3I$ in the headspace of the roots in the presence of $I^-$ by gas chromatography (Saini et al., 1995). In the presence of $^-$SCN, the promoter will activate the expression of *TMT1* gene leading to the production of TMT enzyme. In the presence of $I^-$, TMT will methylate the halide ion producing its methyl halide $CH_3I$. The level of $CH_3I$ is an indication of TMT activity, and therefore, promoter activity.

If the rapid screening with the root-culture system shows that the promoter is able to induce the expression of *TMT1* gene only in the presence of $^-$SCN, the promoter activity could be tested in common transformation protocols such as the floral dip of *A. thaliana* that yields whole transgenic plants. TMT activity could be also tested in the same way by measuring $CH_3I$ level in the headspace of each plant. These transgenic plants would be either growing in a $^-$SCN-containing or $^-$SCN-free media. When $^-$SCN is absent, $CH_3I$ level is expected to be very low compared to its level in the presence of $^-$SCN.

After proving that the $^{-}$SCN-inducible promoter is able to regulate the expression of *TMT1* gene, the next step is to test the efficacy of this system as $^{-}$SCN-inducible selectable marker. The *TMT1* gene alone has been shown to be an effective and reliable selectable marker in many plant transformations, using $^{-}$SCN as the selective agent. The marker gene, regulated by the $^{-}$SCN-inducible promoter, has to be tested for selection in root and plant systems. It can be tested in the same species in which the marker gene alone has been tested: root cultures of basil, carrot, tobacco, nasturtium, potato, green bean, tomato, and sunflower, and in whole plants including the dicots tobacco and *A. thaliana,* and the monocot rice. It would be an advantage to use the same species and compare the effectiveness of the marker alone to that of the $^{-}$SCN-inducible marker. We hope that the inducible marker will have the same or better accuracy than that of the marker. In this way, it would be the first evidence of a marker that can be regulated by its selective agent. A major advantage would be to overcome the concerns about the presence of this marker in transgenic plants after the selection process and the possible side effects that it might have on the plant growth and development.

# 4. Conclusion

This study described the identification and isolation of a possible $^{-}$SCN-inducible promoter. This promoter regulated the expression of a gene that codes for a BSD domain-containing protein in *A. thaliana*. In the presence of $^{-}$SCN, there was an increase in the expression of this gene. Differential display analysis and northern blot analysis showed that this gene is expressed in plants exposed to 1 mM $^{-}$SCN, compared to a very low level of expression in plants growing in normal medium. Another gene was also identified in rice by these two techniques and was induced by $^{-}$SCN, a gene coding for oryzain γ. However, the promoter regulating the expression of this gene was not isolated. For *A. thaliana*, the region upstream of the gene's transcription start site was isolated and amplified. It is expected to contain the promoter region that is inducing the expression of the gene in the presence of the ion $^{-}$SCN. Further approaches would be to measure the promoter activity through the GUS assay. If the promoter could induce the expression of *gusA* gene only in the presence of $^{-}$SCN, then the promoter could be inserted upstream of *TMT1* gene to regulate its expression. The regulation of *TMT1* expression by $^{-}$SCN would allow the development of an inducible selectable marker that is only expressed during the selection process, and then turned off when the selection is achieved.

# 6. References

**Attieh J, Djiana R, Koonjul P, Etienne C, Sparace SA, Saini HS** (2002) Cloning and functional expression of two plant thiol methyltransferases: a new class of enzymes involved in the biosynthesis of sulfur volatiles. Plant Mol Biol **50:** 511-521

**Attieh J, Kleppinger-Sparace KF, Nunes C, Sparace SA, Saini HS** (2000) Evidence implicating a novel thiol methyltransferase in the detoxification of glucosinolate hydrolysis products in *Brassica oleracea* L. Plant, Cell and Environment **23:** 165-174

**Attieh J, Sparace SA, Saini HS** (2000) Purification and properties of multiple isoforms of a novel thiol methyltransferase involved in the production of volatile sulfur compounds from *Brassica oleracea.* Arch Biochem Biophys **380:** 257-266

**Attieh JM, Hanson AD, Saini HS** (1995) Purification and characterization of a novel methyltransferase responsible for biosynthesis of halomethanes and methanethiol in *Brassica oleracea.* J Biol Chem **270:** 9250-9257

**Bertolla F, Kay E, Simonet P** (2000) Potential dissemination of antibiotic resistance genes from transgenic plants to microorganisms. Infect Control Hosp Epidemiol **21:** 390-393

**Chiter A, Forbes JM, Blair GE** (2000) DNA stability in plant tissues: implications for the possible transfer of genes from genetically modified food. FEBS Lett **481:** 164-168

**Clough SJ, Bent AF** (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana.* Plant J **16:** 735-743

**Cuellar W, Gaudin A, Solorzano D, Casas A, Nopo L, Chudalayandi P, Medrano G, Kreuze J, Ghislain M** (2006) Self-excision of the antibiotic resistance gene *nptII* using a heat inducible Cre-loxP system from transgenic potato. Plant Mol Biol **62:** 71-82

**Dale EC, Ow DW** (1991) Gene transfer with subsequent removal of the selection gene from the host genome. Proc Natl Acad Sci U S A **88:** 10558-10562

**Doerks T, Huber S, Buchner E, Bork P** (2002) BSD: a novel domain in transcription factors and synapse-associated proteins. Trends Biochem Sci **27:** 168-170

**Duncan AJ** (1991) Glucosinolates. In toxic substances in crop plants (eds J.P. Felix D'Mello & C.M. Duffus). The Royal Society of Chemistry, Edinburgh 126-147

**Halkier BA, Du L** (1997) The biosynthesis of glucosinolates. Trends in Plant Science **2:** 425-431

**Hare PD, Chua NH** (2002) Excision of selectable marker genes from transgenic plants. Nat Biotechnol **20:** 575-580

**Heinemann JA** (1991) Genetics of gene transfer between species. trends Genetics **7:** 181-186

**Holtorf S, Apel K, Bohlmann H** (1995) Comparison of different constitutive and inducible promoters for the over-expression of transgenes in *Arabidopsis thaliana.* Plant Mol Biol **29:** 637-646

**Jefferson RA, Kavanagh TA, Bevan MW** (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. Embo J **6:** 3901-3907

**Jezek J, Haggett BG, Atkinson A, Rawson DM** (1999) Determination of glucosinolates using their alkaline degradation and reaction with ferricyanide. J Agric Food Chem **47:** 4669-4674

**Keil M, Sanchez-Serrano J, Schell J, Willmitzer L** (1990) Localization of elements important for the wound-inducible expression of a chimeric potato proteinase inhibitor II-CAT gene in transgenic tobacco plants. Plant Cell **2:** 61-70

**Kooshki M, Mentewab A, Stewart ANJ** (2003) Pathogen inducible reporting in transgenic tobacco using a GFP construct. . Plant Science **165:** 213-219

**Kutach AK, Kadonaga JT** (2000) The downstream promoter element DPE appears to be as widely used as the TATA box in Drosophila core promoters. Mol Cell Biol **20:** 4754-4764

**Liang P, Pardee AB** (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science **257:** 967-971

**Liang P, Zhu W, Zhang X, Guo Z, O'Connell RP, Averboukh L, Wang F, Pardee AB** (1994) Differential display using one-base anchored oligo-dT primers. Nucleic Acids Res **22:** 5763-5764

**Miki B, McHugh S** (2004) Selectable marker genes in transgenic plants: applications, alternatives and biosafety. J Biotechnol **107:** 193-232

**Mikkelsen MD, Petersen BL, Olsen CE, Halkier BA** (2002) Biosynthesis and metabolic engineering of glucosinolates. Amino Acids **22:** 279-295

**Mujacic M, Cooper KW, Baneyx F** (1999) Cold-inducible cloning vectors for low-temperature protein expression in *Escherichia coli*: application to the production of a toxic and proteolytically sensitive fusion protein. Gene **238:** 325-332

**Ni X, Hager LP** (1998) cDNA cloning of *Batis maritima* methyl chloride transferase and purification of the enzyme. Proc Natl Acad Sci U S A **95:** 12866-12871

**Rivoal J, Hanson AD** (1994) Choline-O-sulfate biosynthesis in plants (identification and partial characterization of a salinity-inducible choline sulfotransferase from species of *Limonium* (Plumbaginaceae). Plant Physiol **106:** 1187-1193

**Saini HS, Attieh J, Hanson AD** (1995) Biosynthesis of halomethanes and methanethiol by higher plants via a novel methyltransferase reaction. Plant, Cell and Environment **18:** 1027-1033

**Sheen J** (2002) A transient expression assay using *Arabidopsis* mesophyll protoplasts. http://genetics.mgh.harvard.edu/sheenweb/

**Stewart CN, Jr., Richards HAt, Halfhill MD** (2000) Transgenic plants and biosafety: science, misconceptions and public perceptions. Biotechniques **29:** 832-836, 838-843

**Visser RGF, Jacobsen E, Witholt B, Feenstra WJ** (1989) Efficient transformation of potato (*Solanum tuberosum* L.) using a binary vector in *Agrobacterium rhizogenes*. Theor Appl Genet **78:** 594-600

**Wang Y, Chen B, Hu Y, Li J, Lin Z** (2005) Inducible excision of selectable marker gene from transgenic plants by the cre/lox site-specific recombination system. Transgenic Res **14:** 605-614

**Watanabe H, Abe K, Emori Y, Hosoyama H, Arai S** (1991) Molecular cloning and gibberellin-induced expression of multiple cysteine proteinases of rice seeds (oryzains). J Biol Chem **266:** 16897-16902

**Yurimoto H, Komeda T, Lim CR, Nakagawa T, Kondo K, Kato N, Sakai Y** (2000) Regulation and evaluation of five methanol-inducible promoters in the methylotrophic yeast *Candida boidinii*. Biochim Biophys Acta **1493:** 56-63

**Zhang W, Subbarao S, Addae P, Shen A, Armstrong C, Peschke V, Gilbertson L** (2003) Cre/lox-mediated marker gene excision in transgenic maize (Zea mays L.) plants. Theor Appl Genet **107:** 1157-1168

**Zhang X, Henriques R, Lin SS, Niu QW, Chua NH** (2006) Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. Nat Protoc **1:** 641-646